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Proteolytické systémy krevničky střevní *(Schistosoma mansoni)*

Proteolytic systems of the blood fluke, *Schistosoma mansoni*

Disertační práce

Školitel: Mgr. Martin Horn, CSc.

Praha, 2018

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Obsah

Seznam zkratek

Abz	aminobenzoyl
ACC	7-amino-4-karbamoylmethylkumarin
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoridhydrochlorid
AMC	7-amino-4-methylkumarin
ATP	adenosintrifosfát
CA074	L-3-*trans*-(propylkarbamoyl)oxiran-2-karbonyl)-L-isoleucyl-L-prolin
Boc	t-Butyloxykarbonyl,
BPTI	hovězí pankreatický inhibitor proteas trypsinového typu (z angl. "Bovine Pancreatic Trypsin Inhibitor")
Bz	benzoyl
CHO	aldehydová skupina
CMK	chloromethylketon
Col I – IV	kolgen typ I-IV
DMSO	dimethylsulfoxid
DTT	dithiothreitol
E-64	N-[N-(L-3-trans-karboxyoxirin-2-karbonyl)-L-leucin]-agmatin
EDTA	ethylendiamintetraoctová kyselina
ES	exkrečně-sekreční
FPLC	kapalinová chromatografie (z angl. "Fast Protein Liquid Chromatography")
FRET	"Fluroescence Resonance Energy Transfer"
Hb	hemoglobin
HPLC	kapalinová chromatografie (z angl. "High Performance Liquid Chromatography")
HSA	hovězí sérový albumin
HsPOP	lidská prolyloligopeptidasa
HsCC	lidský katepsin C
IC_{50}	koncentrace inhibitoru potřebná k dosažení 50% inhibice enzymu
IgG	imunoglobulin G
K11777	N-methyl-piperazin-Phe-homoPhe-vinylsulfon-fenyl
kDa	kilodalton
LmSAP1	signální peptid z prvoka *Leishamania mexicana* reakce (z angl. "Secreted Acid Phosphatase ")
LHRH	gonadorelin (z angl. "LH-releasing hormone")
Mb	megabáze

MeOSuc	3-Methoxysukcinyl
αMSH	α-melanocyty stimulující hormon
MS	hmotnostní spektrometrie
Nph	nitrofenyl
PCR	polymerasová řetězová reakce (z angl. "Polymerase Chain Reaction")
PDB	"Protein Data Bank", databáze proteinových struktur
PMSF	fenylmethylsulfonyl fluorid
SDS-PAGE	elektroforéza na polyakrylamidovém gelu v přítomnosti SDS
SmAE	asparaginylendopeptidasa z z krevničky *S. mansoni*
SmCalp1	kalpain 1 z krevničky *S. mansoni*
SmCalp2	kalpain 2 z krevničky *S. mansoni*
SmCOX I	cytochromoxidasa I z krevničky *S. mansoni*
SmCB1	katepsin B1 z krevničky *S. mansoni*
SmCB2	katepsin B2 z krevničky *S. mansoni*
SmCC	katepsin C z krevničky *S. mansoni*
SmCD	katepsin D z krevničky *S. mansoni*
SmDPPII	dipeptidylaminopeptidasa II z krevničky *S. mansoni*
SmDPPIII	dipeptidylpeptidasa III z krevničky *S. mansoni*
SmDPPIV	dipeptidylaminopeptidasa IV z krevničky *S. mansoni*
SmCL1/ SmCF	katepsin L1 (katepsin F) z krevničky *S. mansoni*
SmCL2	katepsin L2 z krevničky *S. mansoni*
SmCL3	katepsin L3 z krevničky *S. mansoni*
SmLAP	leucylaminopeptidasa z krevničky *S. mansoni*
SmPCP	prolylkarboxypeptidasa z krevničky *S. mansoni*
SmPOP	prolyloligopeptidasa z krevničky *S. mansoni*
SmSP1-5	serinová proteasa 1-5 z krevničky *S. mansoni*
STI	sójový inhibitor trypsinu (z angl. "Soybean Trypsin *Inhibitor*")
Suc	sukcinyl
SÚKL	Státní ústav pro kontrolu léčiv
TFPAMK	tetrafluorofenoxyarylmethylketon
TLCK	Nα-tosyl-L-lysin chloromethyl keton
TPCK	N-p-tosyl-L-fenylalanin chloromethyl keton
Tris	tris(hydroxymethyl)aminomethan
UCSF	"University of California San Francisco"
UCSD	"University of California San Diego"
VS	vinylsulfon
Z	Benzyloxykarbonyl,
1,10 Ph	1, 10 – phenantrolin

Abstrakt

Schistosomóza je závažné parazitární onemocnění člověka, jehož původcem jsou krevničky, krevní motolice rodu *Schistosoma*. Celosvětově je schistosomózou, která představuje globální světový problém, infikováno přes 200 miliónů lidí a dalších 750 miliónů je vystaveno riziku nákazy. Jediný účinný lék pro léčbu schistosomózy je chemoterapeutikum praziquantel, u kterého je riziko vzniku rezistence. Proteasy krevničky představuji nadějné cílové molekuly pro vývoj nových terapeutických strategií proti schistosomóze. Tato práce se zaměřuje na komplexní charakterizaci proteolytických systémů krevničky *Schistosoma mansoni* a určení jejich role v interakci s hostitelem. Za prvé byly pomocí metod funkční proteomiky popsány hlavní proteolytické aktivity sekretované jednotlivými vývojovými stádii krevničky, která parazitují v těle člověka. Tato analýza prokázala jejich komplexní a specifickou distribuci s převažujícími serinovými a cysteinovými proteasami a metaloproteasami. Za druhé byly pomocí přístupu chemické genomiky identifikovány povrchové a trávicí proteasy krevničky, konkrétně prolyloligopeptidasa a katepsiny typu B, C a D, jako vhodné cílové molekuly pro terapeutickou intervenci. Prolyloligopeptidasa byla biochemicky charakterizována na úrovni rekombinantního proteinu, byly vyvinuty její účinné inhibitory jako templáty pro antischistosomální léčiva a byla navržena biologická role proteasy v modulaci hemostázy hostitele.

Klíčová slova: parazit, krevnička, *Schistosoma mansoni*, proteasa, inhibitor

Abstract

Schistosomiasis is a serious parasitic disease caused by blood flukes of the genus *Schistosoma*. It is a global health problem with more than 200 million people infected and 750 million people at risk. Current therapy relies on a single drug, praziquantel, for which there are concerns of emerging drug resistance. Proteases of schistosoma are promising target molecules for the development of new therapeutic strategies against schistosomiasis. This work focuses on the comprehensive characterization of proteolytic systems of *Schistosoma mansoni* and determination of their role in the interaction with the human host. First, the major proteolytic activities secreted by individual developmental stages of schistosoma that parasitize the human body were classified using functional proteomics. This analysis demonstrated their complex and specific distribution with predominant serine and cysteine proteases and metalloproteases. Second, tegumental and digestive proteases, namely prolyl oligopeptidase and cathepsins B, C and D, were identified by chemical genomics as suitable target molecules for therapeutic intervention. Prolyl oligopeptidase was biochemically characterized using a recombinant protein, its effective inhibitors were developed as templates for antischistosomal drugs, and a biological role of the protease in modulation of host hemostasis was proposed.

Keywords: parasite, blood fluke, *Schistosoma mansoni*, protease, inhibitor

Úvod

Schistosomóza je vážné parazitické onemocnění, které postihuje přes 200 milionů lidí v tropických a subtropických oblastech světa a představuje významný zdravotní i sociálně ekonomický problém (World Health Organization (2.6.2018); (Chitsulo et al. 2000; Steinmann et al. 2006). Schistosomóza je způsobena krevničkami rodu *Schistosoma*. K nákaze člověka dochází v kontaminovaném sladkovodním prostředí, kde se nacházejí infekční larvy cerkárie. Cerkárie aktivně pronikají skrz kůži a jako schistosomuly postupují do krevního řečiště definitivního hostitele. Dospělé krevničky žijí v cévní soustavě hostitele, kde dochází k produkci velkého počtu vajíček. Závažnost onemocnění závisí na délce trvání infekce, na intenzitě nákazy a na imunitním stavu infikovaného jedince. Hlavním patogenním agens onemocnění jsou vajíčka, která kolují v krevním řečišti a usazují se v různých tkáních, kde způsobují prudkou imunitní odezvu organismu spojenou s poškozením orgánů. Dostupnost léčby a potlačení nemoci stále není dostatečné a počet infikovaných lidí není trvale redukován (Chitsulo et al. 2000).

Jediný účinný lék pro léčbu schistosomózy je chemoterapeutikum praziquantel, který se nepřetržitě používá již více než 30 let, proti schistosomóze v současnosti neexistuje žádná dostupná očkovací látka. Proto stále narůstá riziko vzniku rezistence a potřeba vývoje nových alternativních chemoterapeutik a vakcín (Caffrey 2007; Cioli et al. 2004).

Proteolytické enzymy (proteasy) jsou nezbytné pro přežití krevniček v těle hostitele. Jsou zásadní pro invazi do hostitele, migraci, trávení, vývoj, reprodukci a pro modulování fyziologických funkcí a imunitní odpovědi hostitele. Proto jsou stále častěji studovány jako potenciální terapeutické cíle a vakcinační kandidáti.

Tato disertační práce se zabývá proteasami parazita krevničky střevní, *Schistosoma mansoni*. Hlavním cílem je identifikace proteas kritických pro životaschopnost krevničky, jejichž blokování pomocí účinných inhibitorů či vakcín by bylo pro parazita letální. Tyto proteasy představují potenciální molekulární cíle pro léčbu schistosomózy**.**

1. Literární úvod a přehled dané problematiky

1.1. Parazitické krevničky rodu Schistosoma

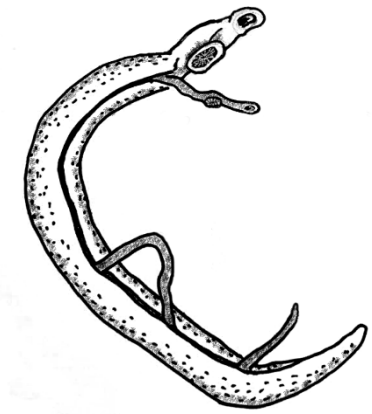
Krevničky (schistosomy) jsou až 2 cm dlouzí parazité, patřící do kmene ploštěnců (Plathelmintes), třídy motolic (Trematoda) (Obr. 1A, str. 6). Jsou to gonochoristé odděleného pohlaví s výrazným pohlavním dimorfismem (Obr. 1B, str. 6), kteří parazitují v cévní soustavě obratlovců a způsobují onemocnění člověka a zvířat zvané schistosomóza. První krevničku (*Schistosoma haematobium*) popsal v roce 1851 v Káhiře německý internista Theodor Bilharz (odtud starší název bilharzie a bilharzióza (Schadewaldt 1962)).

Z rodu *Schistosoma* člověka napadá druh *Schisotosoma haematobium* (krevnička močová, egyptská schistosomóza), *Schistosoma mansoni* (krevnička střevní, americká schistosomóza), *Schistosoma japonicum* (krevnička jaterní) a dále pak i méně známé *Schistosoma intercalatum, Schistosoma mekongi* nebo *Schistosoma malayensis* (Förstl et al. 2003).

A **Taxonomické zařazení**

Nadříše: Eukaryotae
Říše: Animalia
Podříše: Metazoa
Kmen: Platyhelminthes
Třída: Trematoda
Čeleď: Schistosomatidae
Rod: *Schistosoma*
Druh: *Schistosoma mansoni*

B ***Schistosoma mansoni***

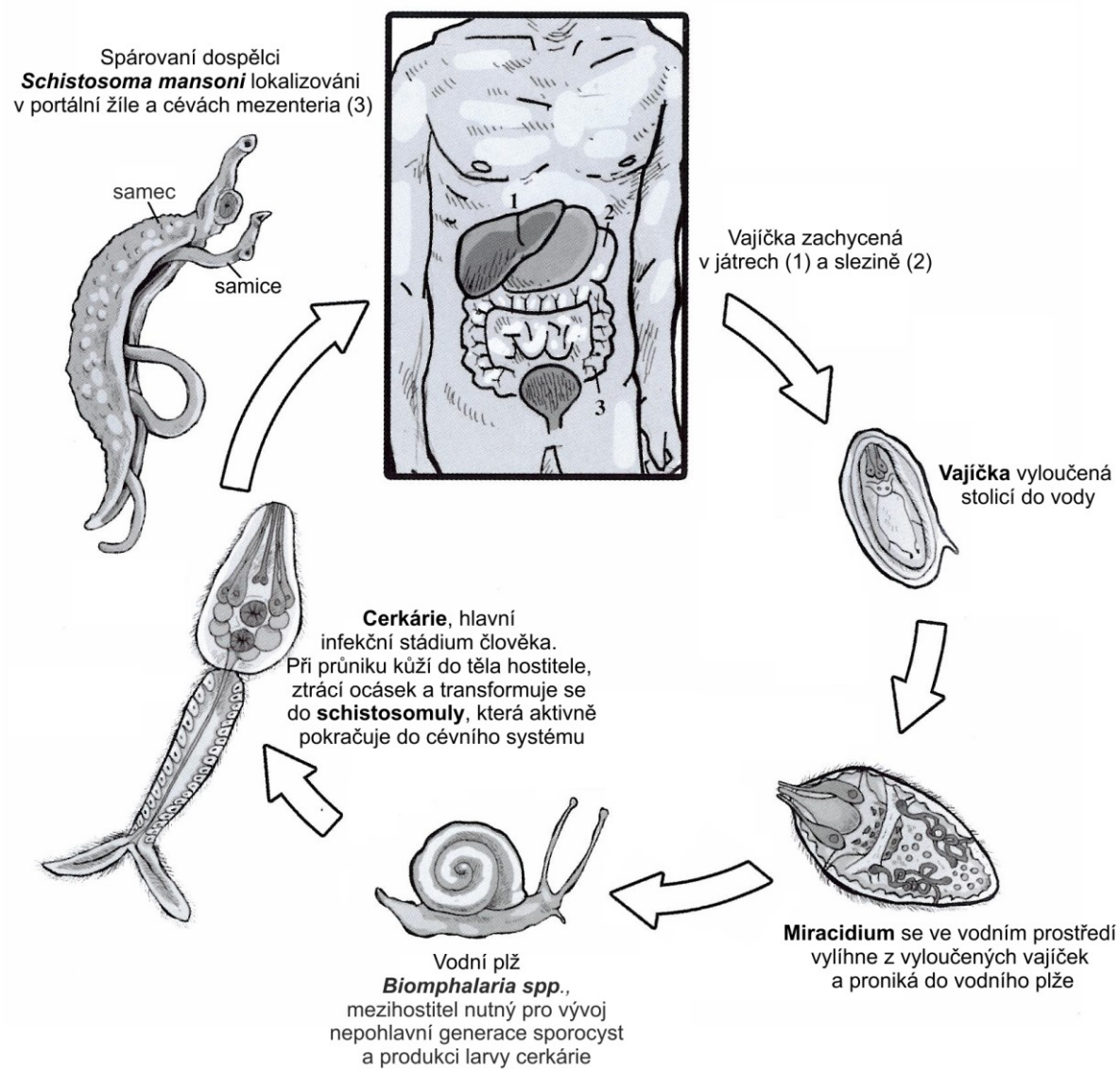


Obrázek 1: A) Taxonomické zařazení druhu S. mansoni (Muller 2002). B) Dospělé krevničky S. mansoni. Patrný je výrazný pohlavní dimorfismus, kdy menší samička je umístěna v canalis gyneacoforus na břišní straně samečka (vlastní zpracování).

1.1.1. Životní cyklus krevniček

Životní cyklus krevniček (Obr. 2, str. 8) zahrnuje pohlavní generaci jedinců vyskytujících se v definitivním hostiteli (člověk) a generaci s nepohlavním rozmnožováním přítomnou v mezihostiteli (sladkovodní plž). K infekci člověka dochází při kontaktu lidské kůže s kontaminovanou vodou, ve které se nacházejí infekční larvy krevničky, cerkárie. **Cerkárie** má charakteristický rozdvojený ocásek (Mair et al. 2003), který při průniku kůží nebo sliznicí hostitele odpadá a larva se přeměňuje na další vývojové stádium zvané schistosomula neboli juvenilní schistosoma. **Schistosomula** proniká přes tkáně do žilního systému a dále je pasivně unášena krevním oběhem do plic, do srdce a do portální žíly, kde v průběhu času roste a dospívá. Po 3-4 týdnech se **dospělci** spárují a sameček přenáší samičku do cév mezenteria obklopujících střeva (*S. mansoni* a *S. japonicum*) nebo do cév urogenitálního traktu (*S. haematobium*), kde samička klade vajíčka. Množství vajíček závisí na druhu krevničky. U samičky druhu *S. haematobium* je denní produkce 20-200 vajíček, u *S. mansoni* více než 300 a u *S. japonicum* až 3000 vajíček. Vajíčka pronikají skrz cévní stěny do močového měchýře, odkud jsou vyloučena močí (*S. haematobium*) nebo do střeva a následně jsou vyloučena stolicí (*S. mansoni* a *S. japonicum*). Značné množství vajíček se zachytí v tkáních, zejména játrech, kde vyvolávají zánět a jsou hlavním patogenním agens onemocnění.

Pokud se vajíčka dostanou do sladké vody, vylíhnou se a uvolní se z nich pohyblivá obrvená larva zvaná miracidium. **Miracidium** vyhledává vhodného plže jako mezihostitele a penetruje do jeho tkání. Výběr plže je specifický pro jednotlivé druhy krevniček, pro *S. haematobium* je to rod *Bulinus*, pro *S. mansoni* různé druhy okružákovitých plžů rodu *Biomphalaria* a pro *S. japonicum* rod *Oncomelania*. Miracidium se ve tkáních plže mění v mateřskou sporocystu, která dává vzniknout dceřiným sporocystám. Ty migrují do trávicích žláz plže a nepohlavním rozmnožováním vznikají tisíce nových sporocyst, jež se po inkubační době mění na larvy cerkárie (Volf et al. 2007). Cerkárie opouštějí tělo vodního plže a pomocí pozitivní fototaxe směřují k hladině, kde je největší pravděpodobnost výskytu vhodného definitivního hostitele (Stirewalt and Dorsey 1974). Podnětem k trvalému přilnutí cerkárie *S. mansoni* ke kůži lidského hostitele jsou lipidové struktury ceramidy (Haas et al. 2008).



Obrázek 2: Životní cyklus krevničky střevní. Detailní popis cyklu je rozepsán v textu. Upraveno podle (Bogish et al. 2012).

1.1.2. Onemocnění schistosomóza

Schistosomóza (bilharzióza, schistosomiáza) je nemoc způsobená parazitickými krevničkami rodu *Schistosoma*. Nemocí je nakaženo přes 200 milionů lidí v 78 zemích světa a více než 750 milionů lidí je ohroženo nákazou. Schistosomóza je po malárii druhé nejzávažnější parazitární onemocnění (Chitsulo et al. 2000). Onemocnění je rozšířené v tropických a subtropických oblastech Afriky, Asie a Jižní Ameriky, a to zejména v chudých částech bez přístupu k nezávadné vodě, potřebné jak pro fyziologické potřeby obyvatel, tak pro jejich každodenní aktivity. Schistosomóza se dle World Health Organization řadí mezi tzv. opomíjené tropické

nemoci („Neglected Tropical Diseases“), skupinu chronických infekcí způsobujících postižení a následně i zhoršení sociálně ekonomických podmínek, především v extrémně chudých venkovských oblastech nebo znevýhodněných městských populacích (Hotez et al. 2007; Hotez and Kamath 2009; Hotez and Fenwick 2009). Dostupnost léčby a potlačení nemoci stále není dostatečné a počet infikovaných lidí není trvale redukován (Chitsulo et al. 2000).

Závažnost onemocnění závisí na délce trvání infekce, na intenzitě nákazy a na imunitním stavu infikovaného jedince. Hlavním patogenním agens jsou vajíčka schistosom, která se usazují v tkáních hostitele. Zde způsobují prudkou imunitní odezvu organismu spojenou s poškozením orgánů.

Průběh nemoci se dělí na akutní a chronickou fázi. Akutní fáze zahrnuje období od penetrace cerkárie do vývoje v dospělé schistosomy a produkce vajíček. Onemocnění může zůstat i velmi dlouho bez jakýchkoli příznaků. V ojedinělých případech a při opakovaných infekcích se v místě proniknutí larvy do kůže může objevit vyrážka.

Čtyři až šest týdnů po nákaze dochází k migraci larev parazitů do žil mezenteria. V této fázi dochází k produkování vajíček schistosom. Symptomy jako horečka, nevolnost, únava, bolest hlavy a svalů, eozinofilie, zvětšená játra a slezina jsou vyvolané imunitní odpovědí na antigeny produkované vajíčky. Akutní fáze nemoci se u jednotlivých nemocných projevuje v různé intenzitě a při zanedbání nebo absenci léčby jsou následky nemoci dlouhodobé. V chronické fázi dochází ke konstantnímu hromadění vajíček ve tkáních různých orgánů, nejčastěji jater a slinivky, kde vyvolávají zánětlivé imunitní reakce vedoucí k tvorbě granulomů. Opakované záněty vedou ke kalcifikaci orgánů a poškození jejich funkce (Roberts and Janovy 2006).

1.1.3. Výskyt krevniček a patologie schistosomózy

Nejběžnějšími druhy parazitujícími u člověka jsou krevnička močová (*S. haematobium*), krevnička střevní (*S. mansoni*) a krevnička jaterní (*S. japonicum*) (Volf, Horák, and kol. 2007).

S. haematobium se vyskytuje v Africe a na Středním východě. *S. haematobium* způsobuje tzv. egyptskou schistosomózu. Při infekci se vajíčka parazita usazují v močovém měchýři hostitele. Typickým projevem nemoci je přítomnost malého množství krve v moči. Sekundárními projevy infekce jsou zánětlivé změny hlavně v urogenitálních oblastech, kde

mohou být příčinou vzniku rakoviny (Volf et al. 2007). V závislosti na rozsahu infekce mohou být zasaženy játra, plíce a mícha hostitele.

S. mansoni se vyskytuje v Africe, na Středním východě, na Karibských ostrovech, v Brazílii, Venezuele a Surinami. *S. mansoni* způsobuje tzv. střevní schistosomózu. Hlavními zasaženými orgány jsou tlusté střevo a játra, kde se následně zvyšuje pravděpodobnost nádorového onemocnění. Léze se objevují i v plicích a v míše.

S. japonicum se vyskytuje v Číně, Indonésii a na Filipínách. *S. japonicum* je původcem jaterní schistosomózy. Po penetraci cerkárií nastává časná fáze infekce, jejíž projevy jsou eozinofilie, erytém a horečka Katayama. Tento druh schistosom způsobuje těžké chronické onemocnění s rozsáhlým poškozením jater, sleziny, střev a plic. Závažnou komplikací jsou léze kolem vajíček zanesených krevním řečištěm do mozku, tzv. cerebrální schistosomóza, která končí často smrtí (Volf et al. 2007).

1.1.4. Diagnostika schistosomózy

Diagnózu schistosomózy může potvrdit serologické vyšetření pomocí ELISA testu, který specificky detekuje schistosomální vaječný antigen (Němečková and Kolářová 2004). Vajíčka schistosom je možno prokázat mikroskopicky 4-6 týdnů po infekci v močovém sedimentu (*S. haematobium*) nebo v preparátu ze stolice (*S. mansoni* a *S. japonicum*).

Patologické změny a ložiska orgánového postižení lze pozorovat klasickými diagnostickými zobrazovacími metodami (ultrazvuk, počítačový tomograf, nukleární magnetická rezonance). Jelikož schistosomóza je parazitární onemocnění, které není endemické v České republice, je pro správnou diagnostiku vedle klinického obrazu důležitá i cestovatelská anamnéza.

1.1.5. Terapie schistosomózy

Již více než 30 let je lékem první volby praziquantel (v ČR dostupný pod komerčním názvem Biltricide a Cesol) (SÚKL (3.6.2018)). Praziquantel vykazuje vysokou účinnost proti všem druhům i kmenům schistosomy a je dobře snášen s minimálními vedlejšími účinky. Při slabších nákazách stačí jediná dávka 40 mg/kg/den, při silnějších nákazách 60 mg/kg ve dvou dávkách během dne. Mechanismus účinku není dosud přesně známý, ale předpokládá se souvislost interakce praziquantelu s Ca^{2+} kanály krevniček (Jeziorski and Greenberg 2006),

což vede ke stahům svaloviny dospělých červů a jejich uvolnění od cévní stěny. Navíc dochází k poškození a vakuolizaci tegumentu, k expozici antigenů na povrch parazita a následnému rozpoznání imunitním systémem hostitele (Thetiot-Laurent et al. 2013; Harnett and Kusel 1986).

Lék není vhodný u pacientů s portální hypertenzí, neboť se dostává nemetabolizovaný do centrální nervové soustavy a působí neuropsychické obtíže (Němečková and Kolářová 2004). Léčebný účinek je u praziquantelu limitován malou účinností proti schistosomulám, proto nejlépe zabírá až po 5. týdnu infekce, kdy dojde k přeměně schistosomul na dospělce (Sabah et al. 1986). Praziquantel je doposud nejúspěšnější chemoterapeutikum pro léčbu schistosomózy. Jelikož se nepřetržitě používá již přes 30 let po celém světě, narůstají obavy z vývoje rezistence (Caffrey 2007; Caffrey 2015). Případy nízké citlivosti vůči praziquantelu již byly zaznamenány (Ismail et al. 1999; Melman et al. 2009; Cioli et al. 2004) a v laboratorních podmínkách na myších se rezistenci podařilo vyvolat (Fallon and Doenhoff 1994).

Dražší alternativou pro léčbu schistosomózy je oxamniquine (Taylor et al. 2015), který je však účinný pouze na druh *S. mansoni*. Lék se podává orálně a zabraňuje syntéze DNA a RNA u parazita. Ačkoliv je léčba efektivní, u léku byly zaznamenány případy rezistence (Cioli et al. 1993). V České republice není tento lék registrován (SÚKL (3.6.2018)).

Proti schistosomóze v současnosti neexistuje žádná očkovací látka (McManus and Loukas 2008; Gryseels 2000). Dospělé krevničky přežívají v krevním řečišti člověka až 30 let, a proto mají dobře vyvinuté strategie pro únik lidskému imunitnímu systému (Boros 1989). Zatím nejúspěšnější kandidát na vakcínu pro urinární schistosomózu byla rekombinantní glutathion-S-transferasa (Sh28GST) (Bilhvax, Eurogenentech, Belgie). Vakcína prošla do třetí fáze klinických studií (Riveau et al. 2012), ovšem studie byla ukončena a výsledky nebyly zveřejněny (ClinicalTrials.gov (2.6.2018)).

Dodnes používané léky pocházejí z výzkumných programů založených na testování různých látek bez znalosti cílových molekul nebo mechanismu působení dané látky (Caffrey 2007). Pro zjišťování účinnosti nových potenciálních inhibitorů proti krevničkám se využívá metoda *in vitro* testování na živých schistosomulách uchovávaných v kultivačních médiích a následné vyhodnocování změny jejich fenotypu. Tato metoda je již částečně automatizovaná a umožňuje efektivní a plošné testování různých látek (Abdulla et al. 2009).

1.2. Proteolytické enzymy

Proteolytické enzymy, zvané také peptidasy či proteasy, štěpí peptidové vazby bílkovin a peptidů. Tento proces se nazývá proteolýza. Předpokládá se, že proteasy se objevily již v raných fázích biologické evoluce, jelikož i ty nejprimitivnější organismy je využívají pro zpracování vnějších proteinových potravních zdrojů a pro štěpení vlastních bílkovin. Dostupné kompletní analýzy genomů ukazují, že 2 % všech genových produktů připadají na proteasy, což je řadí mezi největší funkční skupiny proteinů.

Proteasy náleží v klasifikačním schématu enzymů do třídy hydrolas, využívají tedy molekulu vody k rozštěpení peptidové vazby. Podle nomenklatury proteasových substrátů se aminokyselinový zbytek substrátu účastnící se peptidové vazby svou karboxylovou skupinou označuje jako P_1, aminokyselinový zbytek podílející se svou aminoskupinou se označuje P_1'.

Podle lokalizace štěpného místa se proteasy dělí na endopeptidasy, štěpící uvnitř polypeptidového řetězce a na exopeptidasy, štěpící z N-konce (aminopeptidasy) nebo C-konce peptidového řetězce (karboxypeptidasy). Dipeptidylpeptidasy odštěpují dipeptid z N-konce, peptidyldipeptidasy z C-konce.

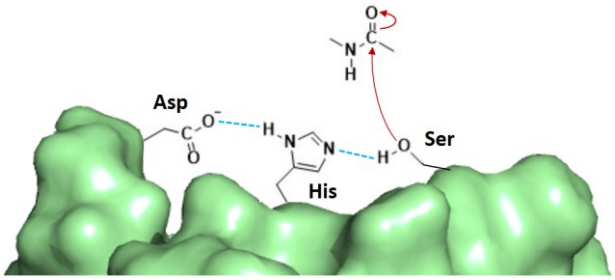
Podle katalytického mechanismu (katalytického aminokyselinového zbytku) rozdělujeme proteolytické enzymy na aspartátové, cysteinové, glutamátové, metaloproteasy, asparaginové, serinové, threoninové proteasy a proteasy s neznámým mechanismem (MEROPS (1.8.2018)). Podle databáze MEROPS jsou proteasy dále rozděleny do klanů a rodin. Klan obsahuje proteasy, které jsou si evolučně příbuzné a mají podobnou terciální strukturu. Klany jsou rozděleny na rodiny podle příbuznosti aminokyselinových sekvencí.

1.2.1. Serinové proteasy

Serinové proteasy obsahují jako katalytický zbytek v aktivním centru aminokyselinu serin. Vyskytují se převážně extracelulárně a preferují slabě alkalické prostředí. Serinové proteasy jsou nejlépe prostudovanou katalytickou třídou proteolytických enzymů.

Při štěpení bílkovin využívají hydroxylu serinového zbytku k nukleofilnímu ataku peptidové vazby. Ke štěpení peptidové vazby dochází nejprve za vytvoření kovalentního acylenzymového meziproduktu, ve kterém karboxylová komponenta peptidového substrátu vytváří ester s hydroxylem katalytického serinu za současného odštěpení C-koncové části peptidu. Ve druhé fázi je pak tento intermediát hydrolyzován molekulou vody a dojde

k uvolnění N-koncového peptidu (Obr. 3, str. 13). Ze serinových proteas se disertační práce bude zabývat proteasami rodiny S1 a rodiny S9, z nich především prolyloligopeptidasou.



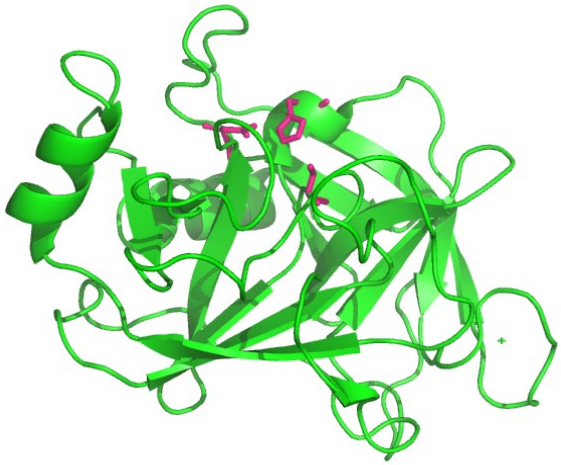
Obrázek 3: Uspořádání aktivního místa serinových proteas. Katalytická triáda se skládá z aminokyselinových zbytků kyseliny asparagové, histidinu a serinu. Hydrolýzu peptidové vazby substrátu začíná nukleofilní atak hydroxylové skupiny serinu na karbonylovou skupinu štěpené peptidové vazby. Detaily jsou uvedeny v textu. Upraveno podle (Neitzel 2010).

1.2.1.1. Serinové proteasy rodiny S1

Rodina serinových proteas S1 patří do klanu PA a obsahuje proteasy zapojené do mnoha kruciálních procesů, ve kterých je vyžadována modifikace nebo degradace proteinů. Obecně se jedná o endopeptidasy nezbytné při lidském vývoji, fyziologii, trávení, pro přirozenou imunitu a invazi patogenů do organismu (Rawlings et al. 2016; Page and Di Cera 2008). Katalytická triáda, která je v sekvenci v pořadí His-Asp-Ser, charakterizuje všechny serinové proteasy z klanu PA (Page and Di Cera 2008).

Proteasy rodiny S1 se syntetizují jako inaktivní prekurzory (zymogeny, proenzymy) obsahující N-koncovou prodoménu. Aktivace je řízena odštěpením prodomény, po kterém dochází ke konformační změně struktury a tvorbě aktivního enzymu (Rawlings et al. 2016). Délka prodomény na N-konci se významně liší od několika až po stovky aminokyselin. Tato prodoména obvykle po odštěpení zůstává vázána disulfidovým můstkem s katalytickou doménou a může obsahovat i jiné neproteolytické, často vazebné domény (Barett et al 2004).

Na základě substrátové specificity v pozici P_1 (Schechter 2005) lze hlavní typy aktivit proteas rodiny S1 rozdělit na (1) chymotrypsinovou s preferencí pro objemné hydrofobní aminokyselinové zbytky (Trp, Tyr, Phe), (2) elastasovou, preferující malé alifatické zbytky (Val, Leu, Ala) a (3) trypsinovou, upřednostňující bazické zbytky Arg nebo Lys (Barett et al 2004; (Rawlings et al. 2016).



Obrázek 4: Prostorový strukturní model lidského trypsinu (PDB kód: 1H4W). Katalytická triáda trypsinu (His57, Asp102 a Ser195) je zobrazena růžově.

Mezi biologicky významné serinové proteasy z chymotrypsinové rodiny S1 patří například trypsin (Obr. 4, str. 14), chymotrypsin, pankreatická elastasa, enteropeptidasa, katepsin G a myeloblastin.

Konzervovaná struktura rodiny S1 (Obr. 4, str. 14) se skládá ze dvou protilehlých šestivláknových domén ß-barelů. Katalytická triáda His, Asp a Ser se nachází na hranici mezi β-barely. Vazebná podmísta rozhodující o substrátové specificitě proteas rodiny S1 mají stejnou strukturu, ale liší se charakteru S_1 podmísta, které je určující pro vazbu substrátu v poloze P_1. Záporně nabitý Asp189 ve spodku S_1 vazebného podmísta trypsinu váže kladně nabité zbytky Arg a Lys v P_1 pozici substrátu (trypsinový typ), zatímco nepolární Ser189 v chymotrypsinu je zodpovědný za vazbu hydrofobních aminokyselin (chymotrypsinový typ).

1.2.1.2. Serinové proteasy rodiny S9

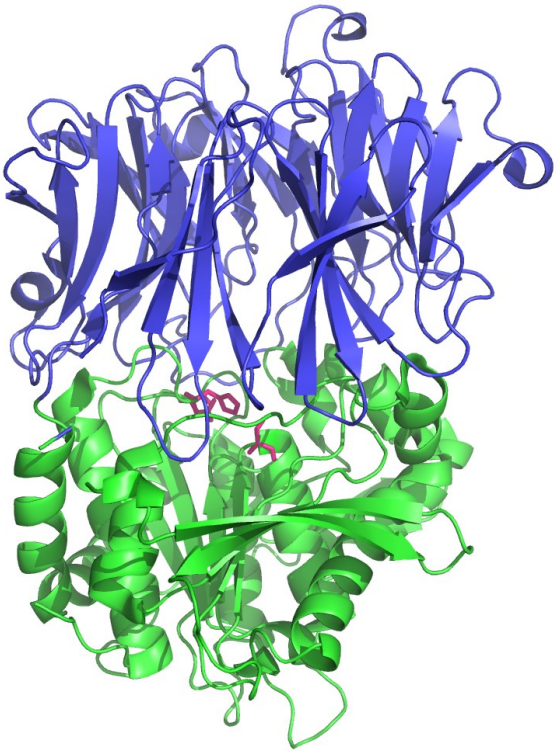
Rodina serinových proteas S9 patří do klanu SC, který je charakteristický katalytickou triádou v pořadí Ser-Asp-His v primární sekvenci (Szeltner et al. 2002). Dalšími obecnými rysy těchto proteas je α/β hydrolasový motiv katalytické domény, která se skládá z osmi vláken středově stočených β-listů obklopených na obou stranách osmi α-helixy, a schopnost štěpit substrát mezi aminokyselinovými zbytky Pro-Xaa (Xaa – jakákoliv aminokyselina). Proteasy rodiny S9 mají molární hmotnost okolo 75 kDa, což je přibližně třikrát víc než proteasy rodiny S1 (např. trypsin a subtilisin (25-30 kDa)) (MEROPS (1.8.2018)). Jsou to jednořetězcové multidoménové proteiny typicky složené z proteasové domény s katalytickými

zbytky na C-konci řetězce, jejichž aktivní místo je blokováno doménou cylindrického tvaru zvanou β-propeler lokalizovanou na N-konci řetězce (Obr. 5, str. 15). β-propeler se skládá ze sedmi nebo osmi opakujících se β-listů, z nichž každý je tvořen čtyřmi antiparalelními peptidovými řetězci. Listy jsou paprsčitě stočeny a vytvářejí ve svém středu tunel. β-propeler stericky blokuje přístup do aktivního místa enzymu a propouští substráty o velikosti menší než 3 kDa (Kiss et al. 2004). Rodina S9 zahrnuje prolyloligopeptidasy, oligopeptidasu B, dipeptidylpeptidasy IV, acylaminoacylpeptidasy a glutamylpeptidasy.

1.2.1.3. Prolyloligopeptidasa (E.C.3.4.21.26)

Prolyloligopeptidasa (POP) byla poprvé identifikována v roce 1971 v lidské děloze jako enzym štěpící oxytocin (Barrett et al. 1982). Dnes jsou popsány tři savčí typy POP – v cytoplazmě (Kato et al. 1980), séru (Dresdner et al. 1982) a v buněčných membránách (Gotoh et al. 1988). Ačkoliv je POP velmi dobře charakterizována strukturně i enzymologicky, její fyziologická funkce nebyla dosud vyřešena. Dostupné informace uvádějí, že POP se účastní mnoha biologických procesů při degradaci peptidových hormonů a neuropeptidů, které jsou úzce spojovány s poruchami paměti, depresemi (Maes et al. 1995) a Alzheimerovou chorobou (Toide et al. 1997), také se podílí na regulaci krevního tlaku (Welches et al. 1993).

Lidská POP štěpí hormony obsahující prolin jako vasopresin a oxytocin (Garcia-Horsman et al. 2007), neuropeptidy ovlivňující paměť a učení jako substanci P a thyrotropin (Blumberg et al. 1980; Yoshimoto et al. 1981) a další substráty jako bradykinin, angiotensin a endorfin (Wilk 1983). Enzymová aktivita POP byla také nalezena v krevní plazmě savců. Jedinou POP, schopnou degradovat proteinové substráty, je POP z parazita *Trypanosoma cruzi* (nazývaná též Tc80), která je schopná degradovat i lidský kolagen typu I a IV (Bastos et al. 2010).



Obrázek 5: Prostorový strukturní model lidské prolyloligopeptidasy (PDB kód: 3DDU). Aminokyselinové zbytky 73 – 427 tvoří tzv. „β–propeler" doménu (modře), která stéricky blokuje přístup do aktivního místa, kde se nachází katalytická triáda (Ser, His, Asp) znázorněna růžově, proteasová doména tvořená aminokyselinovými zbytky 428 – 72 je znázorněna zeleně (Polgar 1992).

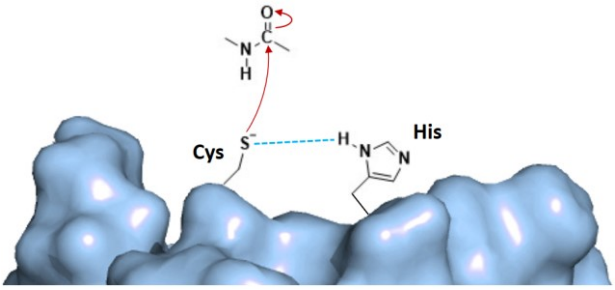
Lidská POP (Obr. 5, str. 15) je tvořena 710 aminokyselinovými zbytky, má válcovitý tvar a jako zástupce rodiny S9 má charakteristickou α/β hydrolasovou doménu, která se nachází na C-konci proteinu a skládá se z motivu sekundárních struktur α/β/α (Fulop et al. 1998). Doména obsahuje katalytickou triádu (Ser, His, Asp) a je kovalentně připojená na β-propeler (Fulop et al. 1998). β–propeler je doména specifická pro POP, má charakteristickou strukturu ze 4 antiparalelních β – listů 7 x se opakujících. Listy jsou paprsčitě stočeny a vytvářejí v jejich středu tunel. β–propeler funguje jako brána ke katalytickému místu enzymu, která propouští substráty o velikosti maximálně 30 aminokyselinových zbytků (Fulop et al. 1998).

Mnoho navržených inhibitorů POP je substrátovými analogy, které jsou charakteristické aminokyselinovým zbytkem prolinu v pozici P_1. Karboxylová skupina prolinu je nahrazena za elektrofilní skupinu karbonitrilu, aldehydu nebo ketonu (Bal et al. 2003). Inhibitory lidské POP jsou v současné době zkoumány jako léčiva u několika neurologických poruch, jako je deprese, Alzheimerova choroba a amnézie. Mnoho inhibitorů

POP je v předklinických a klinických studiích jako nová nootropika (Lawandi et al. 2010). POP je také potenciálním cílem pro léčbu celiakie, zánětlivého onemocnění tenkého střeva způsobené nestráveným lepkem, který je bohatý na prolin (Bastos et al. 2010).

1.2.2. Cysteinové proteasy

Cysteinové proteasy, nazvané podle katalytického zbytku cysteinu v aktivním centru (Obr. 6, str. 17), jsou přítomny zpravidla intracelulárně a vyžadují pro svou aktivitu kyselé prostředí. Jsou hojně zastoupené v různých typech živých organismů včetně člověka, antropotropních virů, bakterií a parazitů. Lidské cysteinové proteasy jsou uvnitř buněk v lyzozomech (katepsiny) i v cytoplazmě (kalpainy, kaspasy). V závislosti na sekvenční homologii lze cysteinové proteasy rozdělit do 91 rodin, kdy nejpočetnější a nejlépe popsanou je rodina C1, rodina papainu (MEROPS (1.8.2018)). Disertační práce se dále bude zabývat významnými zástupci rodiny papainu: katepsinem B a katepsinem C.



Obrázek 6: Uspořádání aktivního místa cysteinových proteas. Katalytická dyáda se skládá z aminokyselinových zbytků cysteinu a histidinu. Thiolová skupina cysteinu je deprotonována a vytváří thiolátimidazolový iontový pár s histidinem. Hydrolýza peptidové vazby substrátu začíná nukleofilním atakem thiolové skupiny cysteinu na karbonyl štěpené peptidové vazby. Histidin stejně jako u serinových proteas slouží jako donor protonu. Upraveno podle (Neitzel 2010).

1.2.2.1. Cysteinové proteasy klanu CA, rodiny C1 (rodiny papainu)

Proteasy rodiny papainu jsou syntetizované jako pre-pro-enzymy obsahující signální sekvenci, propeptid a zralý enzym. Signální sekvence zajišťuje transport biosyntetizovaného proteinu přes membránu endoplasmatického retikula a poté je odštěpena. Propeptid pomáhá při správném sbalování proteasy a podílí se na jejím řízeném transportu do lysozomů. Váže se do aktivního místa v molekule zymogenu v obrácené orientaci než substrát a zabraňuje tak

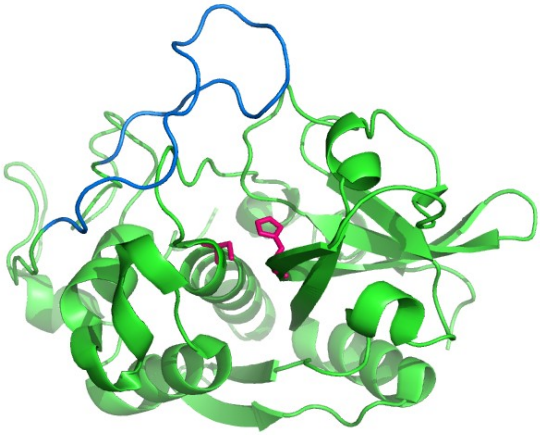
nežádoucí proteolytické aktivitě enzymu v době jeho transportu nebo skladování (Sajid a McKerrow, 2002). Strukturním znakem proteas rodiny papainu jsou levá (L) a pravá (P) doména, které jsou orientovány do písmene V. V L doméně převažují α-helixy, zatímco R doména je tvořena motivem β-barelu. Mezi nimi se nachází aktivní místo enzymu s katalytickými zbytky Cys a His (Obr. 7, str. 19) (Novinec and Lenarcic 2013).

Významnou skupinou proteas rodiny C1 jsou cysteinové katepsiny. V savčím genomu bylo identifikováno 11 cysteinových katepsinů s mnoha rozličnými funkcemi v řadě biologických procesů. Účastní se proteolytické degradace lysozomálních proteinů, přestavby extracelulární matrix, zpracování hormonů a prezentace antigenů. V případě jejich nesprávně regulace se pak mohou uplatňovat v patologických procesech jako je ateroskleróza, revmatická artritida, osteoporóza a procesech spojených s rakovinným bujením (Novinec and Lenarcic 2013).

Disertační práce se zabývá významnými zástupci klanu CA rodiny papainu: katepsinem C (dipeptidylaminopeptidasa I), který odštěpuje po dvou aminokyselinách z N-konce substrátu a karboxypeptidasou katepsin B, která svou karboxydipeptidasovou aktivitu (odštěpuje po dvou aminokyselinách z C-konce substrátu) kombinuje s aktivitou endopeptidasovou (Jilkova et al. 2014).

1.2.2.2. Katepsin B (EC. 3.4.22.1)

Katepsin B (CatB) je lysozomální cysteinová proteasa rodiny C1 s endopeptidasovou a karboxypeptidasovou (peptidyldipeptidasovou) aktivitou, která odštěpuje dipeptidy z C-konce proteinových a peptidových substrátů. Od ostatních katepsinů rodiny C1 se strukturně liší přítomností unikátní smyčky tzv. „occluding loop“, která se nachází v levé doméně enzymu (Musil et al. 1991) a ovlivňuje jeho substrátovou specificitu (Jilkova et al. 2014) (Obr. 7, str. 19).



Obrázek 7: Prostorový strukturní model lidského katepsinu B (PDB 1CSB) Katepsin B je zobrazen stužkovým modelem zeleně, „occluding loop" je zobrazena modře, katalytické zbytky Cys a His mezi pravou a levou doménou jsou zvýrazněny růžově.

CatB je syntetizován na drsném endoplasmatickém retikulu jako 339 aminokyselin dlouhý pre-pro-enzym složený ze signálního peptidu, propeptidu a zralého enzymu. Po transportu proteinu přes membránu endoplasmatického retikula je odštěpen signální peptid. Během transportu endoplasmatickým retikulem je prokatepsin B (43-46 kDa) glykosylován (vzniká oligosacharidový řetězec mannosového typu) a poté translokován do Golgiho aparátu a lysozomů. Zde dochází k aktivaci, při které je ze zymogenu autokatalyticky odštěpen propeptid. Zralý CatB je dále procesován na těžký (25-26 kDa) a lehký (5 kDa) řetězec, které jsou propojené dvěma disulfidovými můstky (Kirschke et al. 1995; Mort and Buttle 1997).

Savčí CatB vykazují hydrolytickou aktivitu v slabě kyselém prostředí, pH optimum je mezi 5,0-6,0 (Barrett and Kirschke 1981; Kirschke, Barrett, and Rawlings 1995). Z hlediska hydrolytické aktivity, štěpí své substráty v endopeptidasovém i exopeptidasovém módu (Barrett and Kirschke 1981). Exopeptidasová aktivita je umožněna smyčkou "occluding loop", která interaguje s aktivním místem a stericky blokuje vazbu substrátu za S_2' vazebným podmístem, čímž zaručuje peptidyldipeptidasovou aktivitu. Substrátová specifita je určena zejména S_2 vazebným podmístem, kde stejně jako řada ostatních proteas rodiny papainu preferuje velké hydrofobní aminokyseliny (Cezari et al. 2002), ale na rozdíl od ostatních zde akceptuje i arginin (Hasnain et al. 1993). Specifickými ireversibilními inhibitory pro CatB jsou inhibitor CA074 (N-(L-3-transkarboxyoxiran-2-karbonyl)-L-isoleucyl-L-prolin) a jeho deriváty, vytvořené od neselektivního epoxidového inhibitoru cysteinových proteas E-64 (Murata et al. 1991).

Hlavní funkcí CatB je lysozomální degradace proteinů. Další funkcí je proteolytická úprava jiných proteinů např. aktivace metaloproteas, urokinázy a katepsinu D. (Alapati et al. 2014; Vigneswaran et al. 2000). V nádorech je zvýšena exprese CatB, který je sekretován z buněk a má vliv na degradaci extracelulární matrix a invazi tumorů, buněčné signalizaci či na snižování exprese proteasových inhibitorů (Yang et al. 2016). CatB je také zapojen do procesů autofagie a katabolismu u maligních tumorů a také je pravděpodobně zapojen do procesů při specifické imunitní rezistenci (Fais 2007).

1.2.2.3. Katepsin C (EC. 3.4.14.1)

Katepsin C (CatC), také známý jako dipeptidylpeptidasa I je lysozomální cysteinová proteasa rodiny papainu. CatC vykazuje dipeptidylaminopeptidasovou aktivitu, odštěpuje tedy dipeptidy z N-konce proteinových a peptidových substrátů. Substrátová specificita je široká, odštěpuje dipeptidy s výjimkou případů, kdy je blokována aminoskupina N-konce, v sousedství štěpené vazby se nachází Pro zbytek nebo N-koncový aminokyselinový zbytek substrátu je Lys nebo Arg (McGuire et al. 1992). CatC je syntetizován jako jednořetězcový neaktivní zymogen. Obsahuje signální sekvenci, N-koncovou doplňkovou doménu („exclusion domain“), aktivační peptid, těžký a lehký řetězec tvořící katalytickou doménu (Dolenc et al. 1995). Ta je z 30-40 % identická s dalšími proteasami rodiny papainu jako katepsin B, H, K, L a S (Kominami et al. 1992). Doplňková doména je však mezi proteasami rodiny papainu unikátní.

Aktivace proenzymu lidského CatC začíná vyštěpením řetězce aktivačního peptidu, přičemž N-koncové doplňková doména zůstává součástí aktivního enzymu, který je dále fragmentován na lehký řetězec a těžký řetězec. Proteolytické aktivace CatC se účastní další proteasy, pravděpodobně cysteinové proteasy jako jsou katepsiny L a S (Dahl et al. 2001).

Obrázek 8: Prostorový strukturní model lidského katepsinu C (PDB 1K3B). *Monomerní jednotka zralého lidského katepisnu C je tvořena katalytickou doménou papainového typu (zeleně), která se skládá z těžkého a lehkého řetězce, a doplňkové domény (červeně). Katalytické zbytky Cys a His jsou zvýrazněny růžově.*

Na rozdíl od ostatních členů rodiny papainu se CatC vyskytuje v oligomerní formě jako tetramer složený ze čtyř identických podjednotek. Každá podjednotka obsahuje doplňkovou doménu, těžký a lehký řetězec, které skládají katalytickou doménu papainového typu (Obr. 8, str. 21). Tvorba tetramerní struktury probíhá nejdříve vznikem dimerů ze dvou monomerních jednotek proenzymu, tyto dimerní podjednotky následně během aktivace dimerizují za vzniku tetrameru (Turk et al., 2001). Interakce dimer-dimer se účastní volný cysteinový zbytek Cys331 (Horn et al. 2002).

Za exopeptidasovou aktivitu CatC odpovídá doplňková doména (Cigič et al. 2000). Doplňková doména představuje izolovanou strukturu, která je schopná sbalit se nezávisle na zbytku enzymu. Zaujímá strukturu β-soudku složeného z osmi antiparalelních β-listů, které jsou stočené kolem hydrofobního jádra (Turk et al. 2001). Objemná doplňková doména blokuje vazebné podmísto S_3, čímž zabraňuje endopeptidasové aktivitě. Zároveň záporně nabitý N-terminální zbytek Asp1 míří do vazebného podmísta S_2 a působí jako kotva pro kladně nabitý N-konec substrátu (Turk et al. 2001). Na každý monomer připadá jedno aktivní místo. Na rozdíl od řady jiných proteolytických komplexů (např. proteasomu) nemá CatC aktivní místa uvnitř struktury, ale vně (Dolenc et al. 1996).

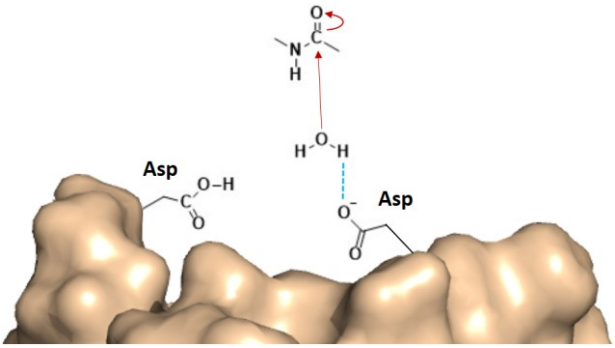
Hlavní funkcí CatC je lysozomální degradace proteinů (Turk et al., 2004). Další funkcí je aktivace proteinů, CatC se podílí na aktivaci elastasy a katepsinu G v neutrofilních buňkách

a chymasy a tryptasy v žírných buňkách. Tyto buňky hrají roli při mnoha zánětlivých onemocněních, jako je revmatoidní artritida, chronické obstrukční plicní onemocnění, zánětlivé střevní onemocnění, astma, sepse a cystická fibróza (Mallen-St Clair et al. 2004).

1.2.3. Aspartátové proteasy

Aspartátové proteasy jsou charakterizovány přítomností dvou katalytických zbytků kyseliny asparagové v aktivním místě enzymu. Peptidová vazba je štěpena přímo molekulou vody aktivovanou vazbou na katalytické aspartáty (Obr. 9, str. 22). Aspartátové proteasy katalyzují hydrolýzu peptidové vazby v mnoha biologických procesech, fungující v trávicím systému u obratlovců jako trávicí enzymy (pepsin), a jako enzymy řídící vylučování vody a iontů ledvinami (renin). Hlavními zástupci aspartátových proteas jsou katepsin D a katepsin E. Obě proteasy působí jako endopeptidasy s maximální aktivitou v kyselé oblasti pH 3,5 až 5,0 (Horn et al. 2014).

CatD je studován jako klíčová proteasa v mnoha patologických procesech jako je Alzheimerova choroba, ateroskleróza a rakovina (Benes et al. 2008). U parazitů, jako např. krevniček, klíšťat nebo zimniček se proteasy typu katepsin D účastní trávení krevních proteinů (Banerjee et al. 2002; Caffrey et al. 2005; Horn et al. 2009; Sojka et al. 2016).

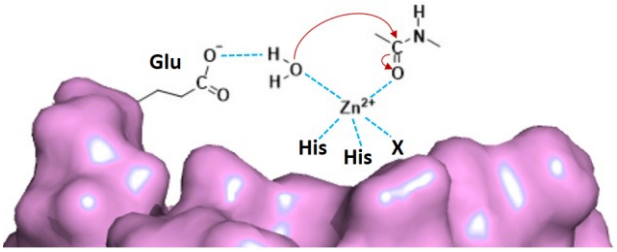


Obrázek 9: Uspořádání aktivního místa aspartátových proteas. Mechanismus aspartátových proteas je založen na acidobazické katalýze, které se účastní dva aspartátové zbytky, jeden aspartátový zbytek se chová jako kyselina a druhý jako báze. Důležitou roli hraje molekula vody, která se váže mezi tyto dva katalytické Asp zbytky. Hydrolýza peptidové vazby substrátu začíná nukleofilním atakem aktivované vody na karbonyl štěpené peptidové vazby. Upraveno podle (Neitzel 2010).

1.2.4. Metaloproteasy

Ze čtyř hlavních typů proteas jsou metaloproteasy nejrozmanitější proteasy, spadající do více než 50 rodin (MEROPS (1.8.2018)). Metaloproteasy využívají při štěpení peptidových vazeb substrátů koordinačního efektu kovového iontu, nejčastěji Zn^{2+} (Obr. 10, str. 23).

Významným zástupcem jsou matrixové metalopeptidasy, extracelulární či membránové peptidasy zapojené především do degradace proteinů mezibuněčné hmoty – extracelulární matrix. Jejich aktivita se zvyšuje zejména v souvislosti s přestavbou nebo obnovou tkání, včetně expanze a diseminace nádorů.



Obrázek 10: Uspořádání aktivního místa metaloproteas. Mechanismus metaloproteas je založen na přítomnosti deprotonovaného glutamátu a za účasti kovového iontu. Mezi bazický zbytek a iont kovu se váže molekula vody. Hydrolýza peptidové vazby substrátu začíná nukleofilním atakem aktivované vody na karbonyl štěpené peptidové vazby. Upraveno podle (Neitzel 2010).

1.3. Proteasy krevničky střevní

1.3.1. Proteasy v genomu *S. mansoni*

Zmapování schistosomálního genomu přineslo obrovské množství dat o potenciálních biologických cílech pro vývoj léčiv proti schistosomóze. Jaderný genom krevničky střevní je tvořen 363 Mb a obsahuje 10852 genů kódujících proteiny (Protasio et al. 2012). Asi 3 % (335 sekvencí) genomu jsou sekvence proteolytických enzymů z pěti tříd proteas (aspartátové, cysteinové, serinové, threoninové a metaloproteasy). Procentuální zastoupení jednotlivých tříd je značně podobné jako v genomu člověka. Nejvýraznějším rozdílem je u krevničky menší zastoupení proteas rodiny S1 chymotrypsinu (16 u krevničky oproti 135 u člověka), které se u člověka podílí na vysoce komplexních a regulovaných proteolytických kaskádách jako je např. srážení krve (Rawlings et al. 2012). Oproti tomu má krevnička více členů metaloproteas rodiny M8 a M13 a cysteinových proteas rodiny C1. Rodina C1 proteas se účastní především trávení krevničky. V genomu bylo rovněž nalezeno 34 proteasových inhibitorů (Kunitz-type, serpiny, cystatiny, alfa-2-makroglobuliny a inhibitory apoptotických proteinů) (Berriman et al. 2009).

1.3.2. Proteolytické systémy krevničky

Proteasy krevniček jsou nezbytné enzymy jak pro larvální, tak pro dospělá stádia. Jsou zásadní pro mnohé z interakcí mezi parazitem a hostitelem, jako jsou invaze, migrace skrze tkáně, degradace proteinů nutných pro výživu, obrana před imunitní odpovědí hostitele a aktivace či modulace zánětlivých procesů (Kasny et al. 2009). U dospělých parazitů hrají roli především dva proteolytické systémy, které hrají klíčovou roli ve vztahu mezi parazitem a hostitelem. Jedná se o proteolytický systém lokalizovaný v trávicí soustavě zodpovědný za trávení pozřených krevních proteinů hostitele a proteolytický systém v povrchové části parazita hrající roli v interakci hostitele s parazitem. Charakterizované proteasy dospělých krevniček *S. mansoni* jsou shrnuty v tabulce 1 (str. 25).

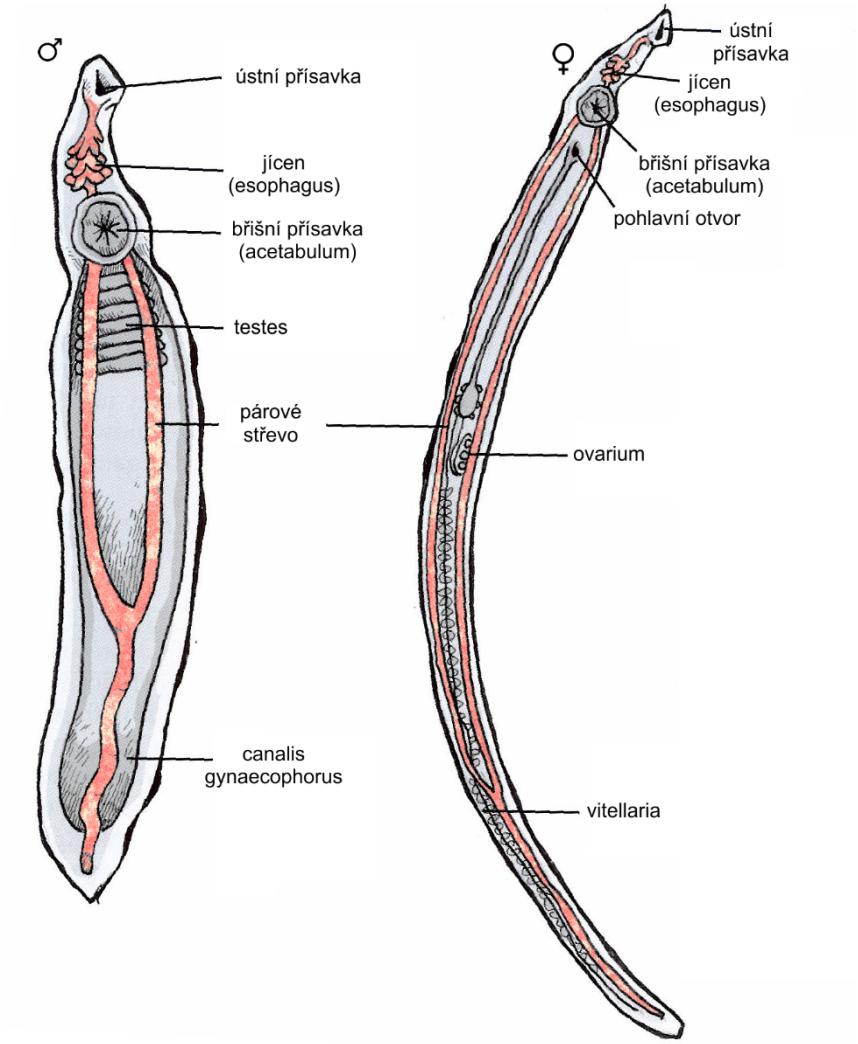
Tabulka 1: Charakterizované proteasy dospělých krevniček S. mansoni. [a] zařazení do rodin dle databáze Merops; [b] endo = endopeptidasová aktivita, exo = exopeptidasová aktivita; karboxy – karboxypeptidasa, amino – peptidasa

Rodina[a]	Proteasa	Označení	Lokalizace	Proteolytická aktivita[b]	Ref.
C1	**Katepsin B1**	SmCB1	Trávicí trakt	endo/exo (karboxy)	(Sajid et al. 2003)
	Katepsin B2	SmCB2	Tegument	exo	(Caffrey et al. 2002)
	Katepsin L1 (katepsin F)	SmCL1/ SmCF	Tegument a trávicí trakt	endo	(Bogitsh et al. 2001)
	Katepsin L2	SmCL2	Reprodukční orgány samiček, acetabulární žlázy	endo	(Michel et al. 1995)
	Katepsin L3	SmCL3	Trávicí trakt	endo	(Dvorak et al. 2009)
	Katepsin C (dipeptidylpeptidasa I)	SmCC	Trávicí trakt	exo (amino)	(Hola-Jamriska et al. 2000)
C2	**Kalpain 1**	SmCalp1	Tegument	endo	(Wilson 2012)
	Kalpain 2	SmCalp2	Tegument	endo	(Wang et al. 2018)
C13	**Legumain (asparaginylendopeptidasa)**	SmAE	Trávicí trakt	endo	(Caffrey et al. 2000)
S1	**Serinová proteasa 1**	SmSP1	Tegument	endo	(Cocude et al. 1999)
S9	**Dipeptidylaminopeptidasa IV**	SmDPPIV	ES produkty	exo (amino)	(Dvorak and Horn 2018)
S9	**Prolyloligopeptidasa**	SmPOP	Tegument	endo	(Fajtová 2011)
S28	**Dipeptidylaminopeptidasa II (dipeptidylpeptidasa 7)**	SmDPPII	ES produkty	exo (amino)	Hall et al. 2011
	Prolylkarboxypeptidasa	SmPCP	ES produkty	exo (karboxy)	Hall et al. 2011
A1	**Katepsin D**	SmCD	Trávicí trakt	endo	(Brindley et al. 2001)
M17	**Leucylaminopeptidasa**	SmLAP	Trávicí trakt	exo (amino)	(McCarthy et al. 2004)
M43	**Dipeptidylpeptidasa III**	SmDPPIII	Homogenát, žádná bližší lokalizace	exo (amino)	(Hola-Jamriska et al. 1999)

1.3.3. Trávicí proteolytický systém krevniček

Trávicí soustava krevničky (Obr. 11, str. 26) začíná ústním otvorem obklopeným silnou ústní přísavkou, následuje jícen (esofagus), v němž začíná samotné trávení lyzí erytrocytů z přijaté krve. Pokračuje párové střevo, které se spojuje asi v polovině délky červa a pokračuje jako jedna trubice. Uvolněný hemoglobin je degradován ve střevě kaskádou proteolytických enzymů (Obr. 12, str. 28). Trávicí trakt je slepý a nestrávené zbytky potravy jsou průběžně vyvrhovány zpět do krve hostitele (Gryseels et al. 2006). Pomocí dominantní břišní přísavky se krevnička pevně uchycuje na stěny cévy.

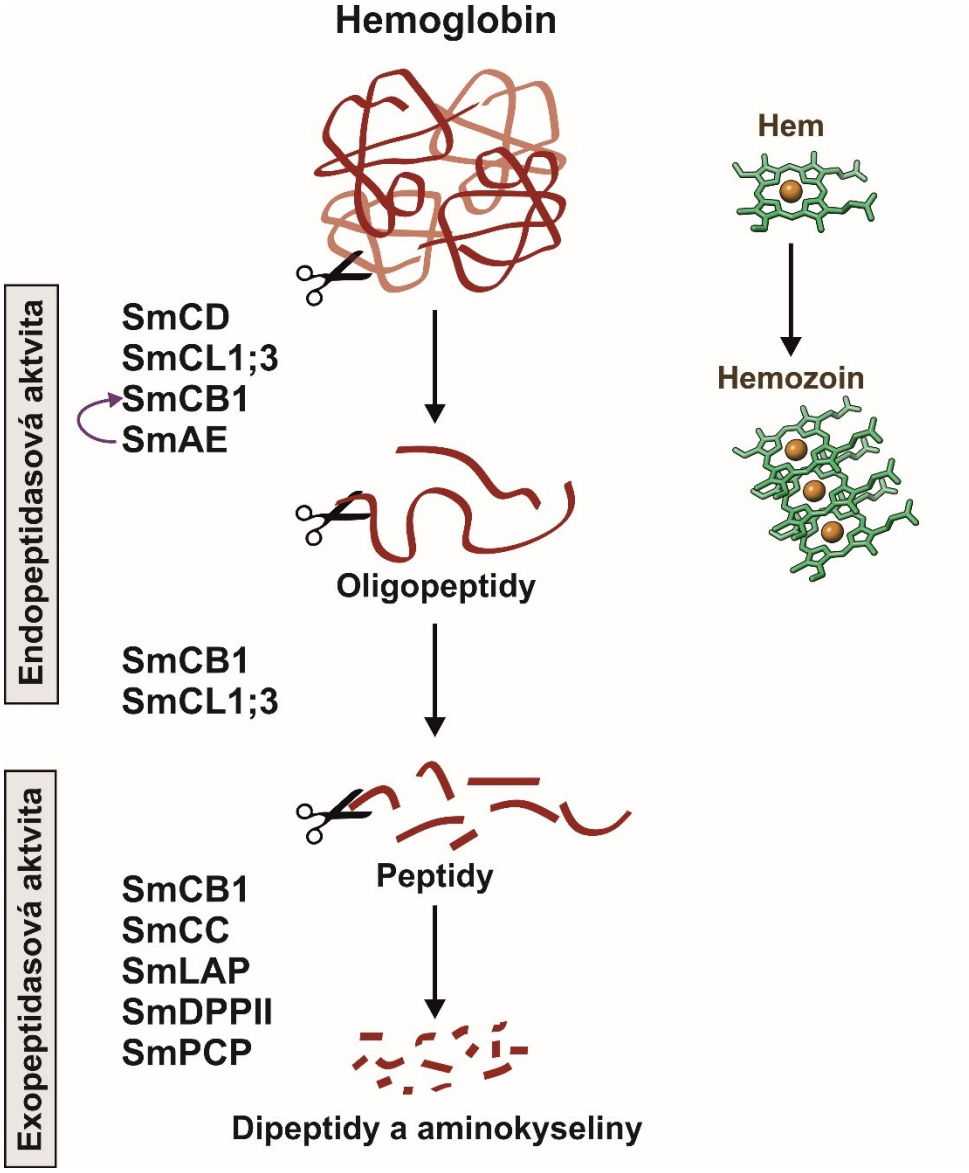
Dospělý samec *S. mansoni* je schopen přijmout 39000 erytrocytů za hodinu, samička, která má větší energetické výdaje spojené s produkcí vajíček, může přijmout až desetinásobek erytrocytů za hodinu (Skelly et al. 2014).



Obrázek 11: Morfologie dospělců S. mansoni. Červeně je vyznačen trávicí trakt u samce a samice dospělé krevničky. (Upraveno podle (Bogish et al. 2012).

1.3.3.1. Trávicí proteasy krevniček

Erytrocyty jsou aktivně nasávány do jícnu parazita, kde začíná jejich rozklad. Přesný mechanismus degradace není přesně známý. Uvolněný hemoglobin se společně s dalšími krevními proteiny dostává do střeva, kde jsou degradovány kaskádou endopeptidas a exopeptidas, nejprve na oligopeptidy, a dále na dipeptidy a jednotlivé aminokyseliny, které jsou následně využity parazitem (Obr. 12, str. 28). Ve střevě krevničky střevní byly pomocí biochemických a imunochemických metod identifikovány cysteinové proteasy: katepsin B1 (SmCB1), katepsin L1 a L3 (SmCL1, SmCL3), katepsin C (SmCC), asparaginylendopeptidasa (SmAE), dále aspartátová proteasa katepsin D (SmCD) a metaloproteasa leucylaminopeptidasa (SmLAP) (Sajid et al. 2003; Hola-Jamriska et al. 1999; Dvorak et al. 2009; Brindley et al. 2001; Caffrey et al. 2000; McCarthy et al. 2004; Caffrey et al. 2004; Delcroix et al. 2006). Při trávení krevních proteinů jednotlivé proteasy kooperují a plní specifické funkce v závislosti na typu proteolytické aktivity. SmCD, SmCL a SmAE působí jako endopeptidasy. SmCB1 vykazuje endopeptidasovou i exopeptidasovou aktivitu (Jilkova et al. 2014). SmCC a SmLAP patří mezi exopeptidasy (Delcroix et al. 2006). V exkrečních produktech gastrointestinálního traktu byla dále popsána prolylkarboxypeptidasa (SmPCP) a dipeptidylpeptidasa II (SmDPPII), které na rozdíl od ostatních proteas jsou schopné štěpit substráty za prolinem (Hall et al. 2011). Mezi proteasami dále existují vztahy na regulační úrovni, jako je role SmAE v aktivaci SmCB1. pH optimum trávicích proteas leží v kyselé oblasti, což bylo stanoveno pomocí syntetických peptidových substrátů i pomocí fyziologických substrátů hemoglobinu a albuminu (Caffrey et al. 2004).



Obrázek 12: Schéma degradace hemoglobinu trávicími enzymy krevničky střevní. Hemoglobin je postupně štěpen endopeptidasami na větší fragmenty, následně na menší fragmenty a oligopeptidy, ty jsou exopeptidasami nakonec štěpeny na dipeptidy a jednotlivé aminokyseliny, které krevnička využívá (Delcroix et al. 2006; Caffrey et al. 2004). Zároveň se z hemoglobinu uvolňuje prostetická skupina hem, který je pro krevničku toxický, a proto je konvertován na nerozpustný krystalický produkt hemozoin. Hemozoin se shromažďuje ve střevě krevničky a je průběžně vyvrhován zpět do krevního řečiště hostitele (Thetiot-Laurent et al. 2013).

1.3.3.2. Katepsin B1 ze *S. mansoni* (SmCB1)

SmCB1 je nejvíce zastoupená cysteinová proteasa ve střevě dospělé krevničky *S. mansoni* (Sajid et al. 2003). Pomocí imunologických a biochemických metod byl lokalizován v lumen střev a v buňkách gastrodermis vystýlající střevní dutinu (Sajid et al. 2003; Ruppel et al. 1987; Chappell and Dresden 1986). Na SmCB1 je soustředěna značná pozornost, protože je považován za klíčovou proteasu v degradaci lidského hemoglobinu (Delcroix et al. 2006). SmCB1 je unikátní v tom, že vykazuje jak endopetidasovou, tak exopeptidasovou (konkrétně peptidyldipeptidasovou) aktivitu. Z hlediska transkripční analýzy je SmCB1 exprimován zejména dospělými krevničkami a larvami schistosomula (Jolly et al. 2007). SmCB1 vykazuje vysokou antigenní schopnost v infikovaných lidech a myších, a je proto potenciální serodiagnostický marker (Ruppel et al. 1985; Klinkert et al. 1991; Losada et al. 2005; Planchart et al. 2007).

SmCB1 je syntetizován jako neaktivní proenzym, ve kterém N-koncový aktivační propeptid stericky blokuje aktivní centrum enzymu. SmCB1 se aktivuje odštěpením N-koncového propeptidu dvěma cestami. Pomocí asparaginylendopeptidasy (Sajid et al. 2003) nebo autokatalyticky v přítomnosti záporně nabitých molekul sulfatovaných polysacharidů (heparin) (Jilkova et al. 2014).

Potlačení exprese SmCB1 v dospělých parazitech *S. mansoni* pomocí RNAi experimentů vyvolalo u parazitů pokles schopnosti štěpit krevní proteiny a bylo docíleno zpomalení růstu krevniček (Delcroix et al. 2006; Correnti et al. 2005).

Inhibitor vinylsulfonového typu K11777, navržený proti cruzainu, cysteinové protease rodiny papainu z parazita *Trypanosoma cruzi* způsobujícího Chagasovu chorobu (McKerrow et al. 2009; McKerrow 2005), byl testován na myším modelu schistosomózy. Výsledky testu prokázaly, že opakované podávání tohoto inhibitoru myším infikovaným larvami *S. mansoni* snižuje patologické stavy způsobené parazitem. Byl redukován počet parazitů a jejich vajíček a následně i postižení jater a sleziny. Jako hlavní molekulární cíl inhibitoru K11777 byla identifikována proteasa SmCB1, a tím validována jako cílová molekula pro vývoj nových antischistozomálních léčiv (Abdulla et al. 2007). Další důkaz byl prokázán pomocí sady inhibitorů odvozených od struktury K11777, které byly testovány *in vitro* jako inhibitory rekombinantního SmCB1 a *ex vivo* na živých schistosomulách v médiu. Výsledky testů prokázaly korelaci inhibičních parametrů a mortality vyvolané u živých parazitů (Jilkova et al. 2011).

1.3.3.3. Katepsin C ze *S. mansoni* (SmCC)

SmCC je cysteinová proteasa, která byla identifikována v gastrodermis dospělých krevniček *S. mansoni* (Bogitsh and Dresden 1983). Předpokládá se, že SmCC se účastní zejména poslední fáze rozkladu krevních proteinů, kdy odštěpuje dipeptidy z peptidových fragmentů substrátu (Delcroix et al. 2006). Aktivita SmCC z dospělých jedinců *S. mansoni* má pH optimum při pH 5,5, přičemž samice krevniček rodu *Schistosoma* mají vyšší specifickou aktivitu než samci, což je v souladu s intenzivní trávicí proteolýzou u samic (Hola-Jamriska et al. 1999).

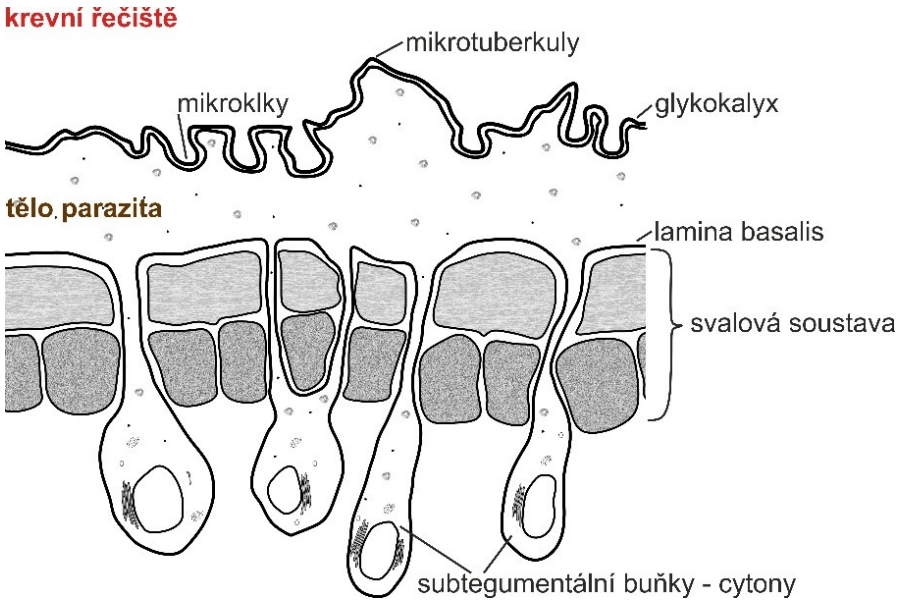
Porovnání sekvence krysího, lidského, myšího, hovězího, psího a dvou schistosomálních katepsinů C prokázalo, že enzym je velmi homologní (Hola-Jamriska et al. 1998). SmCC na rozdíl od lidského a hovězího katepsinu C neobsahuje cysteinový zbytek C331, o kterém se předpokládá, že napomáhá tvořit tetramerní strukturu enzymu (Horn et al. 2002). SmCC nebyl dosud detailně prozkoumán z biochemického a strukturního hlediska.

1.3.4. Tegument krevniček

Úspěšnost schistosom jako patogenů je částečně zapříčiněna díky jedinečné a komplementární rovnováze mezi aktivním získáváním živin prostřednictvím trávicího traktu a zároveň přes povrch těla, tzv. tegument. Jako tegument u krevničky označujeme několik μm tenkou syncytiální vrstvu buněk, která pokrývá celé tělo parazita a odděluje tak jeho vnitřní prostřední od vnějšího (Obr. 13, str. 31). Vnější vrstva tegumentu obsahuje dvě těsně přiléhající lipidové dvojvrstvy (McLaren and Hockley 1977), které zajišťují ochranu před hostitelským imunitním systémem. Tegument je také nezbytný pro výživu z hostitelské krve (Skelly et al. 1994; Skelly and Wilson 2006; Dalton et al. 2004). Vstřebávání přes tegument je převládající cesta absorpce glukózy pro získávání ATP u obou pohlaví. Je pozoruhodné, že samice krevničky požívají méně glukózy, ale konzumují větší množství aminokyselin (proteinů) než samci. Předpokládá se, že pro získávání ATP samice využívají syntézu z uhlíkové kostry aminokyselin. Tato hypotéza ještě nebyla ověřena, ale požadované transaminasy a aminotransferasy nutné k této přeměně jsou zakódovány v genomu schistosom.

Tegument také obsahuje akvaporiny, integrální membránové proteiny umožňující řízený transport vody. Akvaporiny jsou nezbytné pro vylučování (Kloetzel and Lewert 1966) a osmoregulaci parazita (Skelly et al. 2014). V tegumentu se nachází dotykové senzory (Gobert et al. 2003; Jones et al. 2004) a molekulární receptory pro monitorování hladin

hostitelských hormonů, jako je například inzulin hostitele (Dissous et al. 2006). V mezenterických žilách se parazit dokáže maskovat před imunitním systémem pomocí pravidelné obnovy tegumentu, ke které dochází průměrně každých 5 dní (Githui et al. 2009).



Obrázek 13: Schéma tegumentu (povrchu) dospělé krevničky. Vlastní povrch parazita tvoří bezjaderná vrstva, z níž do nitra těla vybíhají tenké cytoplasmatické spoje. Ty jsou lemovány mikrotubuly a prostupují vrstvami lamina basalis a podpovrchové svaloviny, a napojují se na části syncytia obsahující jádro, tzv. subtegumentální buňky. Na povrchu je přítomný glykokalyx a samotný povrch je zvětšen výběžky mikrotuberkulů a mikroklků. Tegument je metabolicky velmi aktivní a slouží nejen k trávení a přenosu živin (např. sacharidů či mastných kyselin), ale i k ochraně parazita před vnějším prostředím, včetně složek imunitního systému hostitele (Volf et al. 2007).

1.3.4.1. Tegumentální proteasy krevniček

Proteomická analýza tegumentu identifikovala řadu unikátních a biologicky důležitých proteinů, jakožto nových potenciálních terapeutických cílů pro vývoj vakcín (Loukas, Tran, and Pearson 2007). Proteasy krevničky lokalizované v tegumentu jsou obecně méně prozkoumané a jedná se zejména o proteasy serinové a cysteinové. V tegumentu byly identifikovány cysteinové proteasy kalpain 1, kalpain 2, katepsin B2, katepsin L1/F a serinová proteasa 1. Jejich přesná funkce není známa.

Katepsin B2 (SmCB2) byl lokalizován v tegumentu a parenchymu dospělců. Předpokládá se, že SmCB2 se může podílet na zpracování tegumentálních proteinů, odbourávání endocytovaných proteinů a procesech spojených s obranou parazita před imunitním systémem hostitele (Caffrey et al. 2002).

Tegumentální cysteinové proteasy kalpain 1 a 2 (SmCalp1, SmCalp2) dokáží štěpit kininogen a fibronektin a tím pravděpodobně ovlivňují vasodilataci cév hostitele, brání srážení krve a vznik zánětu *in vivo* (Wang et al. 2018). SmCalp1 je testován jako vakcinační kandidát (pod názvem Sm-p80) (Jamieson 2016).

Schistosomální katepsin L1 (SmCL1) známý také pod názvem katepsin F (SmCF) (Brady et al. 1999) byl nalezen v gastrodermis parazita a imunolokalizován v subtegumentální oblasti. Předpokládá se, že SmCL1 štěpí imunoglobuliny a tím se účastní obrany před imunitním systémem hostitele (Bogitsh et al. 2001).

Serinová proteasa 1 (SmSP1) byla detekována v exkrečně-sekrečních produktech schistosomul a na povrchu samčího tegumentu (Cocude et al. 1999). SmSP1 je proteasa kalikreinového typu, podobně jako SmCalp1 a SmCalp2 procesuje kininogen na bradykinin a tím reguluje vaskulární funkce hostitele.

2. Cíle práce

Disertační práce se zabývá proteolytickými enzymy z krevničky střevní (*Schistosoma mansoni*), která způsobuje závažné parazitární onemocnění schistosomózu. Proteasy krevniček jsou důležité pro všechna stádia parazita žijící v lidském hostiteli. Hrají roli v interakci mezi parazitem a hostitelem, jako jsou invaze do těla hostitele, migrace tkáněmi, trávení proteinů, potlačení imunity a modulace homeostázy hostitele.

První část disertační práce se zabývá studiem **interakcí krevničky s lidským hostitelem**. Těchto interakcí se významně účastí exkrečně sekreční (ES) produkty parazita. Cílem výzkumu je analyzovat složení proteolytického systému sekretovaného parazitem *S. mansoni*.

Jednotlivé dílčí cíle jsou následující:

1. Příprava ES produktů vývojových stádií krevničky střevní žijících v lidském hostiteli.
2. Mapování hlavních typů proteolytických aktivit v ES produktech pomocí moderních funkčně-proteomických metod a jejich klasifikace.
3. Bioinformatická analýza a analýza exprese hlavních proteas v ES produktech.

Druhá část disertační práce se věnuje vyhledání a funkční charakterizaci proteas jako molekulárních cílů pro **vývoj antischistosomálních léčiv** a identifikaci jejich účinných inhibitorů představujících potenciální léčiva proti schisostosomóze.

Jednotlivé dílčí cíle jsou následující:

1. Identifikace povrchových a trávicích proteas parazita jako cílových molekul pomocí chemicko-genomického přístupu.
2. *In vitro* analýza účinnosti inhibitorů v kinetickém testu s rekombinantními proteasami.
3. *Ex vivo* testování biologické účinnosti inhibitorů na živých larvách *S. mansoni*.

3. Materiál a metodika

Tato kapitola podává obecný přehled o základní metodice, vybavení a použitém materiálu, detailní informace jsou uvedeny v jednotlivých přiložených publikacích. Metody z nepublikovaných výsledků (kapitola 4.3.2.) jsou podrobně rozepsány.

3.1. Materiál a laboratorní vybavení

Disertační práce vznikla s využitím vybavení a přístrojů v laboratořích Ústavu organické chemie a biochemie AV ČR, v.v.i. a ve spolupráci s domácími i zahraničními výzkumnými institucemi.

Vývojová stádia krevničky střevní poskytla University of Califonia, San Franciso (US), University of California, San Diego (US) a 1. lékařská fakulta Univerzity Karlovy. Laboratoře udržují životní cyklus parazita *Schistosoma mansoni* (Puerto Rico).

Sady inhibitorů poskytli M. Bogyo - Stanford University School of Medicine (US); A. Renslo, W. Roush – University of California San Francisco (US); M. Gütschow - Universität Bonn (Německo); W. Gerwick – Scripps, San Diego (US); P. Majer a M. Hradilek – ÚOCHB AV ČR.

Knihovny substrátů poskytl A. O´Donoghue – University of California San Diego (US)

Králičí polyklonální protilátky proti SmPOP připravila a poskytla Moravian Biotechnology, ČR.

3.2. Metodika

V disertační práci byly využity tyto základní metody:

Metody molekulární biologie

Klonování do plasmidu pET101/D-TOPO. Transformace do buněk *E. coli*, rekombinantní exprese v buňkách *E. coli.*

Biochemické metody

Elektroforetická separace proteinů pomocí SDS-PAGE a detekce proteinů na membráně metodou Western blot, immunodetekce na blotu, chromatografická purifikace proteinů

pomocí FPLC, separace peptidů pomocí RP-HPLC, analýza fragmentace proteinů a peptidů proteasami metodami hmotnostní spektrometrie a N-koncové sekvenování.

Enzymologické metody

Měření aktivity enzymů pomocí syntetických fluorogenních a FRET substrátů, stanovení kinetických parametrů pro inhibitory (IC_{50}). Určení substrátové specificity proteas multiplexovou metodou.

Biologické metody

Test *ex vivo* účinnosti inhibitorů na schistosomulách či dospělcích *S. mansoni* v kultivačním médiu a vyhodnocování indukovaných změn fenotypů, sběr exkrečně-sekrečních (ES) produktů vývojových stádií krevničky.

Příprava rekombinantního SmCC

Pro produkci SmCC byl využit rekombinantní systém *L. tarentolae* (Jena Bioscience), který zaručuje správné sbalení a správné posttranslační modifikace proteinu. Úsek obsahující gen pro SmCC byl amplifikován metodou PCR ze schistosomální cDNA pomocí Phusion High-Fidelity DNA Polymerasy a přímého (tcggtcgacGCTGATACTCCTGCTAACTGTACTTA) a reverzního (aaggctagctAACACCGGATCAAAACGTACA) priméru s restrikčními místy pro endodukleasy SalI a NheI (podtrženo). Amplifikovaný úsek DNA byl vložen do plasmidu pLEXSY-sat2 ve čtecím rámci s N-terminální signální sekvenci LmSAP1, která zaručuje exprimování proteinu do média, a s histidinovou kotvu na C-konci. Konstrukt, jehož sekvence byla ověřena DNA sekvenováním, byl do prvoka *L. tarentolae* trasformován elektroporací podle firemního návodu. SmCC byl produkován kultivací *L. tarentolae* 48 h za třepání 120 rpm při 26 °C a purifikován z expresního média odsolením na koloně Sephadex G-25 (objem 470 ml, průměr 3,2 cm, výška 60 cm; GE Healthcare Life Sciences) ekvilibrované 20 mM Tris-HCl pH 7,0 a purifikací pomocí Ni^{2+} chelatační chromatografie (kolona Hi-Trap IMAC FF, GE Healthcare Life Sciences) za nativních podmínek v 50 mM fosfátovém pufru + 300 mM NaCl, pH 7,4. Navázaný SmCC byl eluován gradientem 250 mM imidazolem a zakoncentrována do 20 mM Na-Ac pufru pH 5,5 na koncentrátorku Amicon Ultracel-10K (Milipore).

4. Výsledky

4.1. Přehled výstupů disertační práce

Výsledky této disertační práce jsou shrnuty celkem ve čtyřech publikovaných pracích a ve dvou rukopisech připravovaných k podání do impaktovaného časopisu. Disertační práce obsahuje také data, která doposud nebyla publikována. Publikace na sebe volně navazují a vzájemně se doplňují. V této části je předložen seznam publikací a pro každou z nich je zde uveden podíl studenta na dané práci.

Seznam publikací

Publikace č. 1: Excretion/secretion products from *Schistosoma mansoni* adults, eggs and schistosomula have unique peptidase specificity profiles.

Dvořák, J., **Fajtová, P**., Ulrychová, L., Leontovyč, A., Rojo-Arreola, L., Suzuki, B. M., Horn, M., Mareš, M., Craik, C. S., Caffrey, C. R., and O'Donoghue, A. J. (2016); *Biochimie* 122, 99-109

Publikace se věnuje identifikaci a charakterizaci proteolytických aktivit v ES produktech vývojových stádií krevničky.

Můj podíl na práci spočíval v kultivaci vývojových stádií krevničky střevní žijících v lidském hostiteli a v přípravě jejich ES produktů. Dále jsem v těchto produktech analyzovala aktivity proteas pomocí kinetického testu s FRET substráty a multiplexové metody s MS detekcí.

Publikace č. 2: Trypsin- and chymotrypsin-like serine proteases in *Schistosoma mansoni* – 'The undiscovered country'

Horn, M., **Fajtová, P**., Arreola, L. R., Ulrychová, L., Bartošová-Sojková, P., Franta, Z., Protasio, A. V., Opavský, D., Vondrášek, J., McKerrow, J.H., Mareš, M., Caffrey, C.R., and Dvořák J. (2014); *PLoS Negl. Trop. Dis.* 8. e2766

Publikace je věnována identifikaci, anotaci, expresní analýze a charakterizaci aktivit serinových proteas rodiny S1 parazita *S. mansoni*.

Můj podíl na práci byla příprava ES produktů a proteinových homogenátů, dospělých parazitů, schistosomul a vajec *S. mansoni,* ve kterých byly analyzovány enzymové aktivity serinových proteas rodiny S1 pomocí sady fluorogenních substrátů.

Publikace č. 3: SmSP2: A serine protease secreted by the blood fluke pathogen *Schistosoma mansoni* with anti-hemostatic properties

Leontovyč A., Ulrychová L., O'Donoghue A. J., Vondrášek J., Marešová L., Hubálek M., **Fajtová P.**, Chanová M., Jiang Z., Craik C. S., Caffrey C. R., Mareš M., Dvořák J., Horn M. (2018); *PLoS Negl. Trop. Dis.*, 12, e0006446

Publikace se věnuje podrobné biochemické funkční charakterizaci serinové proteasy z parazita *S. mansoni* (SmSP2).

Můj podíl na práci bylo určení substrátové specificity rekombinantní SmSP2 pomocí multiplexové metody a porovnání se substrátovou specificitou lidského trypsinu.

Publikace č. 4: Prolyl oligopeptidase from the blood fluke *Schistosoma mansoni*: from functional analysis to anti-schistosomal inhibitors

Fajtová, P., Stefanic, S., Hradilek, M., Dvořák, J., Vondrášek, J., Jílková, A., McKerrow, J.H., Caffrey, C.R., Mareš, M., and Horn, M. (2015); *PLoS Negl. Trop. Dis.*, 9, e0003827

Publikace se věnuje biochemické charakterizaci prolyloligopeptidasy SmPOP ze *S. mansoni*, navržení její biologické role a vývoji a testování SmPOP inhibitorů.

Můj podíl na práci zahrnoval: rekombinantní expresi a purifikaci SmPOP; určení substrátové a inhibiční specificity SmPOP, analýzu degradace proteinových substrátů, peptidových hormonů a neuropeptidů; určení inhibičních konstant IC_{50} pro sadu komerčních a navržených inhibitorů, *ex vivo* testování vlivu inhibitorů na parazitické krevničky a vyhodnocování změny fenotypu parazita, přípravu manuskriptu.

Seznam připravovaných manuskriptů

Připravovaný rukopis č. 1: Functional and binding mode analyses of potent vinyl sulfone inhibitors identify druggable hot spots in schistosomiasis Cathepsin B1 target

Jílková, A., Rubešová, P., Fanfrlík, J., **Fajtová, P**., Řezáčová, P., Brynda, J., Lepšík, M., Horn, M., Caffrey, C.R., and Mareš, M. (in preparation 2018) *ACS Infec. Dis.*

Publikace se věnuje profilování sady vinylsulfonových inhibitorů SmCB1 a analýze jejich vazebného módu pomocí krystalových struktur a kvantově-chemických výpočtů.

Můj podíl na práci spočíval v analýze sady vinylsulfonových inhibitorů pomocí *ex vivo* experimentů na parazitických krevničkách a vyhodnocení indukovaných změn fenotypu tohoto parazita.

Připravovaný rukopis č. 2: *Schistosoma mansoni* cathepsin C (SmCC): SAR analysis of TFPAMK-inhibitors and their anti-schistosomal activity

Fajtová, P., Horn, M., Jílková, A., Oupicová, I., Illichová, H., Deu, E., O'Donoghue, A. J., Caffrey, C.R., Bogyo, M., Mareš, M. (in preparation 2018) *PLoS Negl. Trop. Dis.*

Připravovaná publikace se věnuje biochemické charakterizaci katepsinu C ze *S. mansoni* (SmCC) a testování SmCC inhibitorů.

Můj podíl na práci spočíval v rekombinantní expresi a purifikaci SmCC, *ex vivo* testování vlivu inhibitorů SmCC na parazitické krevničky a vyhodnocování změny fenotypu parazita.

4.2. Mapování proteolytických aktivit sekretovaných krevničkou při interakci s lidským hostitelem

4.2.1. Identifikace tříd proteas v exkrečně-sekrečních produktech jednotlivých vývojových stadií schistosom žijících v lidském hostiteli

Dosavadní výzkum proteas krevniček se soustředil na anotaci jejich sekvencí jako součást globálních genomových (Berriman et al. 2009; Andrade et al. 2011; Tsai et al. 2010; Young et al. 2012) nebo transkriptomických studií (Verjovski-Almeida et al. 2003), proteomickou identifikaci proteas v extraktech vývojových stádií či jejich exkrečně-sekrečních (ES) produktech (Braschi et al. 2006) a na funkční charakterizaci jednotlivých proteas (Dvorak et al. 2009; Caffrey et al. 2002; Sajid et al. 2003). Proteomické analýzy ukazují, že ES produkty obsahují celou řadu molekul, včetně proteas, které hrají nezastupitelnou roli při interakci parazita s hostitelem (McKerrow et al. 2006). Komplexní analýza proteas v ES produktech zahrnující i testování jejich enzymatických aktivit doposud nebyla provedena.

Publiklace č. 1 se věnuje mapování a klasifikaci proteolytických aktivit v ES produktech klíčových vývojových stádií *Schistosoma mansoni*, parazitujících v lidském těle. K tomuto účelu byly sebrány a analyzovány ES produkty (1) pomocí kinetického testu s FRET („Förster resonance energy transfer") substráty a (2) multiplexovou substrátovou knihovnou s MS detekcí (tzv. multiplexová metoda). Do sekvence FRET substrátů je integrována donorová skupina fluoroforu a skupina akceptoru s absorbčním spektrem překrývajícím emisní spektrum donoru. Fluorescence intaktní molekuly je slabá, rozštěpením peptidové vazby mezi skupinou donoru a akceptoru se intenzita fluorescence zvyšuje. Každý

FRET substrát se skládal ze 7- nebo 8-merního peptidu ohraničeného 2,4-dinitrofenyl-L-lysinem na C-konci (akceptor) a 7-methoxykumarin-4-octovou kyselinou nebo 7-methoxykumarin-4-ylacetyl-L-lysinem (donor) na N-konci.

Multiplexová metoda je metoda ke stanovení časově závislého profilu substrátové specificity analyzovaných proteas (O'Donoghue et al. 2012). Jedná se o kombinaci řízené enzymové reakce s LC-MS/MS detekcí. Základem je knihovna 124 oligopeptidů o 14 aminokyselinách sloužící jako substráty pro analyzované proteasy. Fragmentační místa jsou identifikována metodou LC-MS/MS a výsledná data zobrazující substrátovou specificitu v pozicích P_4-P_4‘ jsou vizualizována pomocí ICE-log.

Pro klasifikaci proteasových aktivit byly tyto metody použity v kombinaci se specifickými inhibitory různých tříd proteas: E-64 pro cysteinové proteasy, AEBSF pro serinové proteasy a 1, 10 – phenantrolin inhibitor pro metaloproteasy. Tento postup umožnil určit zastoupení jednotlivých tříd proteas v ES produktech v vývojových stádiích krevničky vyskytujících se v lidském hostiteli a popsat celkovou substrátovou specifitu proteasových aktivit v pozicích P_4-P_4’ (Obr. 14, str. 41).

Každé vývojové stádium *S. mansoni* produkovalo odlišnou sadu aktivních proteas. Výsledky ukazují, že proteasy sekretované schistosomulami výrazně upřednostňovaly degradaci substrátů na C-konci aminokyselinových zbytků Arg nebo Lys (v P_1 vazebné pozici) (obrázek 14A, str. 41). V pozici P_2 byl nejčastěji preferován aminokyselinový zbytek Gln a v podmístě P_1’ Ser nebo Arg. Ostatní pozice nevykazovaly výraznou specificitu. To naznačuje přítomnost jedné hlavní proteasy v ES produktech schistosomul.

Na rozdíl od schistosomul byla mnohem větší promiskuita štěpení u ES produktů dospělců a vajec, projevující se variabilní a ne příliš výraznou substrátovou specificitou v různých pozicích. To naznačuje, že zde dochází k sekreci několika různých typů enzymů. Proteasy v ES produktech dospělců přednostně štěpily aminokyselinové zbytky Arg nebo Trp v pozici P_1 a preferovaly Ser nebo Arg na P_1’. V ostatních pozicích byla velká variabilita preferovaných aminokyslin. Podobně u proteas v ES produktech vajec je výrazná specificita vidět jen v pozici P_1, kde jsou upřednostňovány aminokyselinové zbytky Arg a Gln v P_1’ pro Arg (Obr. 14A, str. 41).

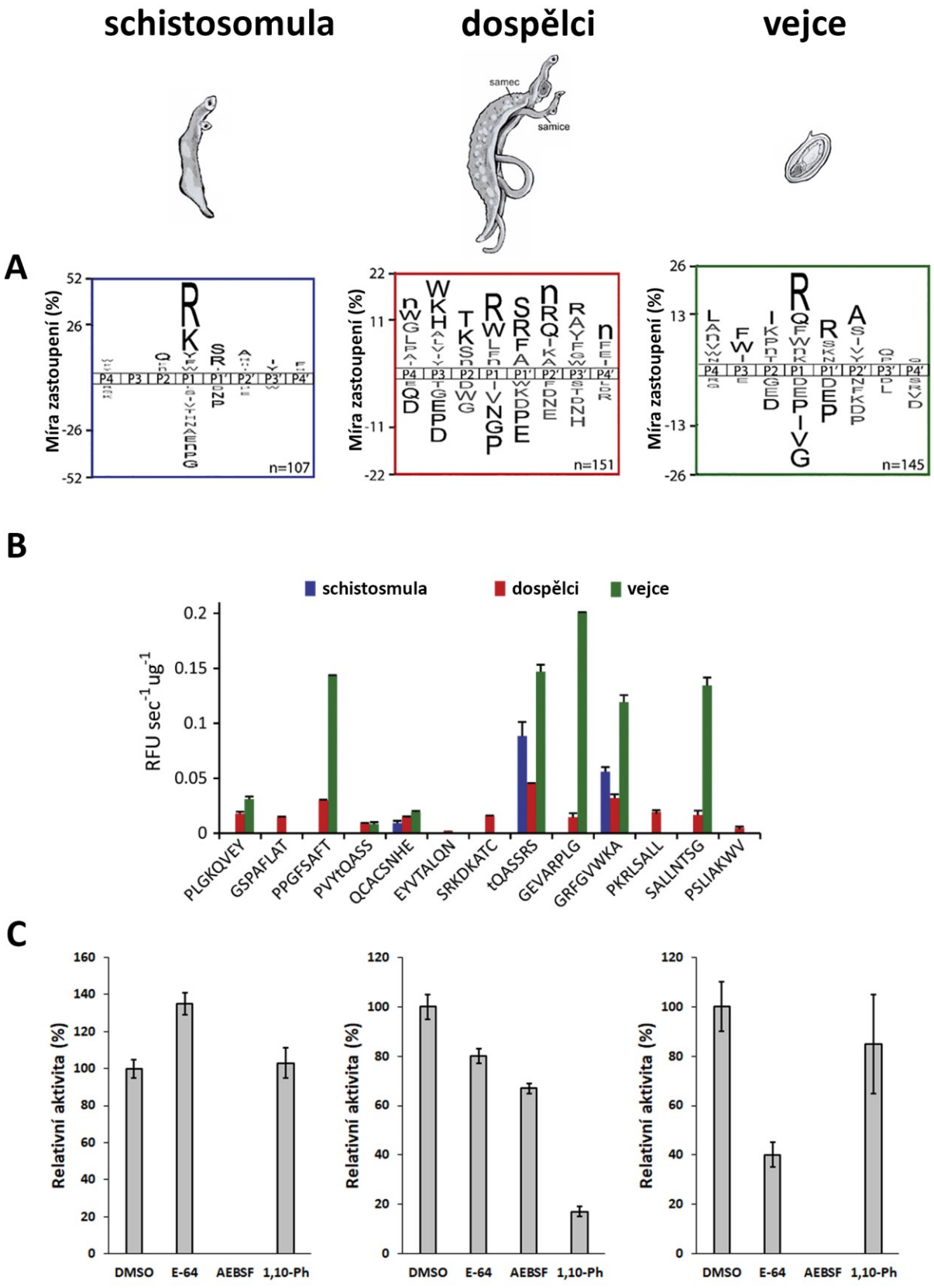
Dále byla testována sada FRET substrátů (Obr. 14B, str. 41) pro určení optimálního substrátu k další analýze aktivit a určení převažujících tříd proteas pro jednotlivá stádia v testu se specifickými inhbitory. Jako optimální substrát pro všechna stádia byl identifikován substrát tQASSRS (t = tert-butyl glycin). Výsledky ukazují, ze v ES produktech schistosomul

převažují serinové proteasy. V kombinaci s výsledky substrátové specificity (Obr. 14A, str. 41) to naznačuje, že se jedná o jedinou serinovou proteasu s trypsinovou aktivitou (tj. štěpcí. za Arg, Lys). Naproti tomu jak v ES produktech dospělců tak ES produktech vajec jsou za fyziologických podmínek zastoupeny tři hlavní třídy proteas – serinové, cysteinové a metaloproteasy. U dospělé krevničky převažují metaloproteasy a serinové proteasy, u vajec je výrazná aktivita serinových a cysteinových proteas, které jsou aktivní v neutrálním pH.

4.2.2. Identifikace a analýza serinových proteas rodiny S1

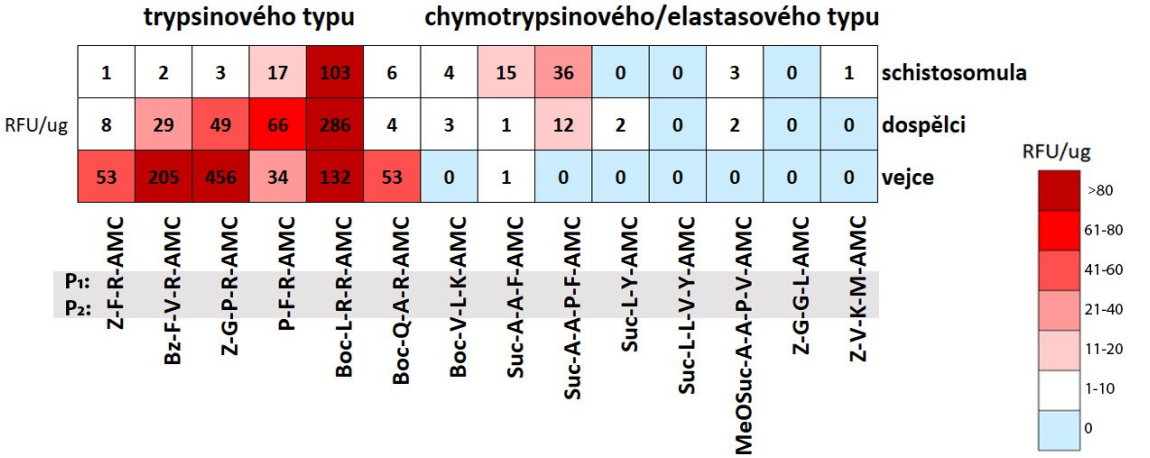
Vzhledem k tomu že v ES produktech všech vývojových stádií byla nalezena výrazná aktivita serinových proteas (Publikace č. 1), byla **publikace č. 2** věnována identifikaci, anotaci, expresní analýze a charakterizaci aktivit těchto sekretovaných serinových proteas. Podle databáze MEROPS (1.8.2018), je nejvýznamnější rodinou serinových proteas (SP) rodina S1 rodina chymotrypsinu. Podle substrátové specificity lze hlavní typy proteolytických aktivit rodiny S1 rozdělit na (1) aktivitu chymotrypsinového typu, která v pozici P_1 preferuje hydrofobní aminokyseliny, (2) elastasového typu, která v pozici P_1 preferuje malé alifatické aminokyseliny a (3) trypsinového typu, kde jsou v pozici P_1 preferovány bazické aminokyseliny.

Enzymové aktivity serinových proteas (SP) rodiny S1 v proteinových homogenátech dospělých parazitů, schistosomul a vajec *S. mansoni* byly mapovány v kinetickém testu pomocí dvou sad peptidových fluorogenních substrátů (Obr. 15, str. 42). První sada obsahovala substráty obecně používané k stanovení trypsinové aktivity s bazickými aminokyselinami (Arg, Lys) v pozici P_1, druhá sada obsahovala hydrofobní aminokyseliny (Phe, Tyr) nebo alifatické aminokyseliny (Val, Leu, Met) v pozici P_1, obecně se substráty této sady používají k měření chymotrypsinové nebo elastasové aktivity (Harris et al. 2000). Substráty se dále lišily v prodloužení do pozic P_2, P_3 a N-terminální blokovací skupinou. Aktivity byly měřeny v kinetickém testu v přítomnosti inhibitorů E-64 a EDTA pro potlačení enzymových aktivit cysteinových proteas a metaloproteas. Příslušnost naměřených aktivit k proteasové rodině S1 byla dále ověřena jejich citlivostí k malým molekulárním inhibitorům, PMSF a AEBSF, které specificky inhibují tuto rodinu proteas.



Obrázek 14: Analýza proteolytických aktivit v exkrečně-sekrečních produktech vývojových stádií krevničky žijících v lidském hostiteli. A) ICE loga znázorňují substrátovou specificitu v pozicích P_4-P_4' sekretovaných proteas S. mansoni určenou metodou multiplex. ES produkty

schistosomul (modrá), dospělců (červená) a vajec (zelená). ICE logo je grafickým znázorněním míry shodných sekvencí aminokyselin v pozicích substrátu. Relativní velikost písmen udává jejich četnost zastoupení vůči celkovému počtu štěpených substrátů; n je počet štěpených peptidů. B) Stanovení aktivity proteas se sadou FRET substrátů. Aminokyseliny jsou popsány v jednopísmenném kódu (t = tert-butyl glycin). C) Určení tříd proteas v ES produktech vývojových stádií pomocí specifických inhibitorů. Aktivita proteas v ES produktech byla měřena v kinetickém testu se substrátem tQASSRS. E-64: inhibitor cysteinových proteas, AEBSF: inhibitor serinových proteas, 1,10-Ph (1, 10 – phenantrolin): inhibitor metaloproteas, DMSO kontrolní vzorek bez inhibitorů.

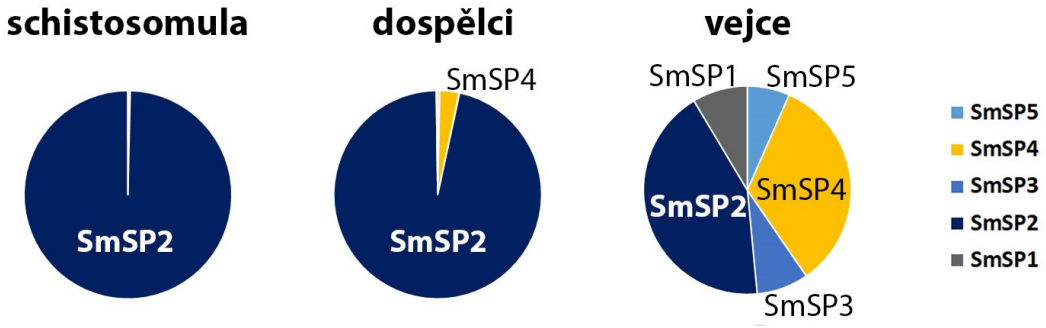


Obrázek 15: Profilování enzymových aktivit serinových proteas v proteinových homogenátech vývojových stadií S. mansoni (dospělci, schistosomuly a vejce). Šedě jsou zvýrazněny substrátové pozice P_2 a P_1. Aktivita SP byla měřena v kinetickém fluorescenčním testu v přítomnosti uvedených substrátů, které jsou specifické pro proteasy trypsinového, chymotrypsinového a elastasového typu. SP aktivity (sensitivní na inhibitory serinových proteas PMSF a AEBSF) byly vyjádřeny v relativních fluorescenčních jednotkách za sekundu (RFU/s) a normalizovány na koncentraci proteinu v jednotlivých extraktech. Barevné kódování pro hodnoty naměřené aktivity ukazuje panel na straně obrázku. Data jsou vyjádřena pomocí teplotní mapy. (Z – Benzyloxykarbonyl, Bz – Benzoyl, AMC – aminomethylcoumarin, Boc – t-Butyloxykarbonyl, Suc – Sukcinyl, MeOSuc - 3-Methoxysucinyl).

Výsledky ukázaly, že v homogenátech všech analyzovaných stádií *S. mansoni* převažují aktivity serinových proteas trypsinového typu. Substráty chymotrypsinového a elastasového typu byly štěpeny jen u schistosomul a velmi slabě u dospělců (Obr. 16, str. 43). Výrazná

enzymová aktivita byla pro všechna stádia nalezena pro substrát Boc-L-R-R-AMC (Obr. 16, str. 43), který byl dále využit k detekci aktivit serinových proteas v ES produktech dospělců, schistosomul a vajec *S. mansoni*. Tato analýza prokázala, že SP jsou krevničkou sekretovány a mohou ovlivňovat interakce s hostitelem.

Bioinformatickou analýzou genomu *S. mansoni* metodou BLASTp za použití sekvence hovězího trypsinu jako templátu bylo identifikováno pět odlišných proteas rodiny S1 nazvaných SmSP1-SmSP5. Tyto proteasy shodně obsahují katalytickou proteasovou doménu na C-konci řetězce, ale mají rozdílnou doménovou strukturou na N-konci řetězce. Pomocí RT-qPCR (Obr. 16, str. 43) byla stanovena míra exprese jejich mRNA v jednotlivých vývojových stádiích. Výsledky analýzy ukázaly, že SmSP1-5 jsou v různé míře zastoupeny ve všech vývojových stádiích *S. mansoni* parazitujících v lidském hostiteli. U schistosomul, dospělců a vajec je nejvíce exprimována SmSP2. Ostatní SmSP byly detekovány v největší míře u vajec.

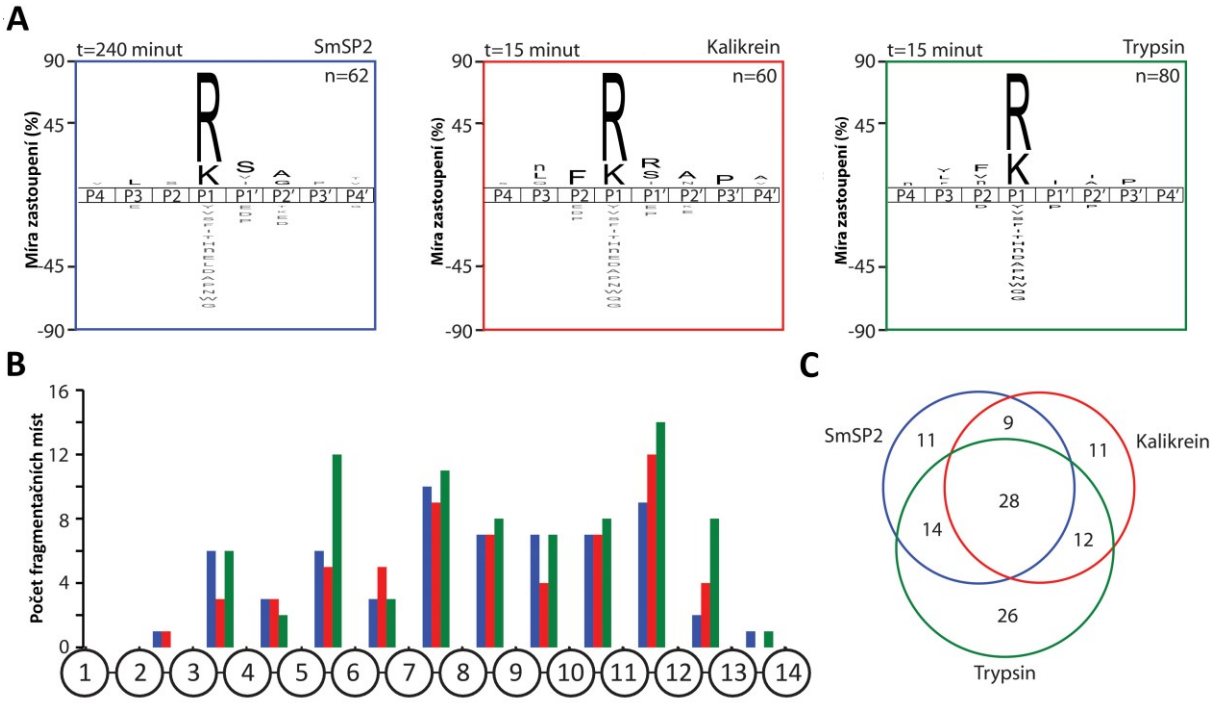


Obrázek 16: Míra exprese mRNA SmSP1-SmSP5 analyzovaná RT-qPCR v různých vývojových stádiích S. mansoni. mRNA úrovně exprese jsou zobrazeny jako procenta genové exprese ve srovnání se schistosomální cytochromoxidasou I (SmCOX I)) v zjednodušených koláčových grafech.

4.2.3. Biochemická charakterizace *S. mansoni* serinové proteasy 2 (SmSP2)

Publikace č. 3 se věnuje podrobné funkčně biochemické charakterizaci SmSP2 nejvíce exprimované protease krevničky z rodiny S1. SmSP2 byla popsána jako multidoménový enzym s katalytickou proteasovou doménou trypsinového typu, která je vázána disulfidem s N-koncovou oblastí podílející se na interakcích protein-protein. SmSP2 byla imunolokalizovaná na povrchu (tzv. tegumentu) dospělých krevniček, odkud je sekretována do prostředí a pravděpodobně se účastní mnoha biologických procesů na rozhraní hostitel-parazit. SmSP2 štěpí pouze vybrané proteiny hostitele, vyštěpuje vazodilatační peptid

bradikinin z prekurzoru kininogenu, fragmentuje fibrinonektin, aktivuje plasminogen na plasmin, naproti tomu hemoglobin a albumin, dva hlavní komponenty krve hostitele, které slouží k výživě krevničky, neštěpí. To naznačuje roli SmSP2 nikoliv v trávení, ale v modulaci hemostázy hostitele.



Obrázek 17: Substrátová specificita SmSP2, lidského plasmového kalikreinu a hovězího trypsinu. Specificita ve vazebných pozicích P_4-P_4' byla určena pomocí multiplexové metody s MS detekcí. A) Výsledná substrátová specificita je zobrazena pomocí ICE loga. Aminokyseliny, které byly nejvíce zastoupeny v dané pozici, jsou zobrazeny nad osou, zatímco aminokyseliny, které byly zastoupené minimálně, jsou zobrazeny pod osou. B) Prostorová distribuce míst štěpení v rámci 14-merního peptidového skeletu. C) Vennův diagram ukazující počet unikátních a společných fragmentačních míst pro SmSP2, kalikrein a trypsin.

Podle charakteru vazebného podmísta S_1 byla SmSP2 klasifikována jako proteasa trypsinového typu. To bylo potvrzeno analýzou substrátové specificity pomocí multiplexové knihovny s MS detekcí. Z výsledků vyplývá, že SmSP2 má striktní substrátovou specificitu trypsinového typu pro bazické aminokyseliny Arg a Lys v P_1 pozici. V pozici P_1' preferuje Ser a v P_2' aminokyselinové zbytky Ala a Gly. V porovnání s hovězím trypsinem a lidským plasmovým kalikreinem (Obr. 17A, str. 44) byl celkový profil štěpení SmSP2 podobný s 28 shodnými fragmentačními místy. Nicméně u trypsinu bylo identifikováno 26 jedinečných štěpů ve srovnání s 11 místy unikátními pro SmSP2 nebo kalikrein (Obr. 17C, str. 44).

Fragmentační místa identifikovaná pro SmSP2 nebyla nalezena na N- nebo C-konci peptidů, což potvrzuje, že SmSP2 patří mezi endopeptidasy (Obr. 17B, str. 44).

4.3. Identifikace a funkční charakterizace proteas krevničky jako molekulárních cílů pro vývoj antischistosomálních léčiv

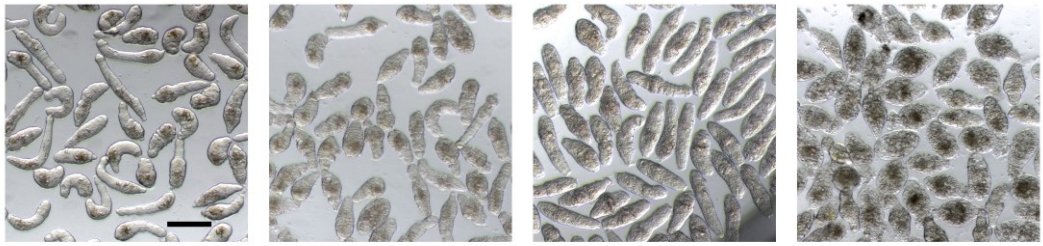
4.3.1. Chemická genomika jako nástroj pro identifikace molekulárních cílů mezi protasami krevničky

Druhá část disertační práce se zabývala vyhledáním sady proteas *S. mansoni* nezbytných pro životaschopnost tohoto parazita, které představují molekulární cíle pro vývoj inhibitorů jako potenciálních antischistosomálních léčiv. K tomuto účelu byla využita chemická genomika, která používá ke studiu biologických procesů malé molekuly schopné modulovat funkce různých genových produktů (nejčastěji proteinů, ale také např. DNA nebo RNA). Zaměřuje se na hledání selektivních molekul ovlivňujících vybraný protein. Takto získaný selektivní nástroj je pak využit ke studiu funkce daného genového produktu – sledování vlivu této látky na fenotyp.

K identifikaci a validaci proteas *S. mansoni* jako molekulárních cílů byly testovány sady inhibitorů z vlastních nebo komerčních zdrojů a inhibitory poskytnuté spolupracujícími institucemi: M. Bogyo (Stanford University School of Medicine, US); A. Renslo, W. Roush (University of California San Francisco, US); M. Gütschow (RFW Universität Bonn, Německo); W. Gerwick (Scripps, San Diego, US); P. Majer a M. Hradilek (ÚOCHB AV ČR). Pro podrobnou studii byla použita kombinace následujících tří přístupů:

a) Biologické *ex vivo* testy na parazitických krevničkách v kultivačním médiu

Biotestem na schistosomulách či dospělcích krevničky v kultivačním médiu bylo sledováno, zda má inhibitor danou biologickou aktivitu a byla určena míra této aktivity (ohodnocená podle závažnosti účinku stupněm 0-4, přičemž stupeň 0 odpovídal parazitům kultivovaných bez inhibitoru a stupněm 4 byl popsán nejzávažnější efekt). Inhibitory byly aplikovány na parazity ve 3 koncentracích (v rozmezí 0,1-10 μM) a vyhodnocování indukovaných změn fenotypů (Obr. 18, str. 46) probíhalo každých 24 h po dva až tři dny.



Obrázek 18: Příklady nejčastěji získaných fenotypů schistosomul indukovaných inhibitorem proteas a jejich označení. Fenotypy vznikají v závislosti na čase a koncentraci inhibitorů a byly klasifikovány do stupňů následujícím způsobem: stupeň 1 – alespoň jeden z fenotypů „dark" (tmavý), „round = R" (všichni stejného tvaru), „slow = S" (pasivní) nebo „overactive = O" (agilní); stupeň 2 – současně dva fenotypy stupně 1; stupeň 3 – současně tři fenotypy stupně 1; stupeň 4 – „degenerated = Deg" (nestrukturovaný tvar parazita, popraskané vakuoly), „dead = D" (neživý); stupeň 0 odpovídá parazitům kultivovaným bez inhibitoru (Kontrola).

b) *In vitro* testování inhibice rekombinantní proteasy

Pro srovnání inhibičních parametrů byly jednotlivé inhibitory testovány v kinetickém aktivitním testu s příslušným rekombinantním enzymem či aktivitou proteasy v homogenátu krevničky a odpovídajícím fluorogenním substrátem. Míra inhibice byla vyjádřena hodnotou IC_{50}, která byla vypočtena nelineální regresí ze závislosti zbytkové aktivity na koncentraci inhibitoru pomocí programu Grafit (Erithacus Software).

c) Ověření suprese daného cíle

Schistosomuly či dospělé krevničky byly po kultivaci s inhibitorem sebrány, propláchnuty kultivačním médiem a homogenizovány. Enzymová aktivita studovaného cílového enzymu byla měřena v kinetickém testu se specifickým substrátem a porovnána vzhledem k aktivitě v homogenátu připraveném z parazitů neošetřených inhibitorem. Kromě cílové proteasy byly v homogenátech sledovány i aktivity dalších proteas ke zjištění míry jejich potlačení.

Připravovaný rukopis č. 1 se zabývá vývojem vinylsulfonových inhibitorů trávicí proteasy katepsinu B1 *S. mansoni* (SmCB1). Obsahuje ukázku chemicko-genomického přístupu a vyhodnocení indukovaných změn fenotypu u schistosomul (Tab. 2, str. 47-48). Účinnost inhibitorů byla testována *in vitro* s rekombinantním SmCB1 a *ex vivo* na parazitických krevničkách.

Tabulka 2: Vinylsulfonové inhibitory SmCB1 a jejich antischistosomální účinek. Inhibice SmCB1 byla určena pomocí kinetického aktivitního měření s rekombinantním SmCB1 a fluorogenním substrátem Z-Phe-Arg-AMC. V biologickém testu na živých schistosomulách v kultivačním médiu byly popsány fenotypy vyvolané vinylsulfonovými inhibitory (aplikovanými v koncentračním rozmezí 1 a 10 μM), které se strukturně lišily v pozici P_3 (struktura inhibitorů není uvedena z důvodu ochrany duševního vlastnictví). Výsledné fenotypy pozorované ve dvou časových bodech byly hodnoceny podle závažnosti účinku v rozmezí 0-4, přičemž stupeň 0 odpovídá fenotypu parazitů kultivovaných bez inhibitoru a stupněm 4 byl popsán nejzávažnější efekt. Pro názornost jsou v tabulce uvedeny indukované fenotypy (Obr. 18, str. 46) a jejich výsledné skóre; n.i. – žádná inhibice.

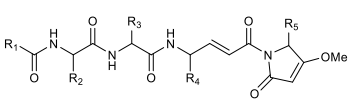
Název inhibitoru	**Inhibice SmCB1 IC_{50} (nM)**	**Indukované fenotypy**				**Stupeň závažnosti fenotypu**			
		10 μM		**1 μM**		**10 μM**		**1 μM**	
		1 d	**2 d**	**1 d**	**2 d**	**1 d**	**2 d**	**1 d**	**2 d**
CB1-1	0.24 ± 0.03					**0**	**0**	**0**	**0**
CB1-2	0.61 ± 0.05		Deg			**0**	**4**	**0**	**0**
CB1-3	1.50 ± 0.13	Deg	Deg			**4**	**4**	**0**	**0**
CB1-4	2.55 ± 0.20	Deg	Deg			**4**	**4**	**0**	**0**
CB1-5	6.00 ± 0.89	S	R, S, Deg	S	R, S, Dark	**1**	**3**	**1**	**3**
CB1-6	7.50 ± 0.58		Deg, R, S		Deg	**0**	**4**	**0**	**4**
CB1-7	13.2 ± 1.3	S	Dark, R		Dark	**1**	**2**	**0**	**1**
CB1-8	39.2 ± 2.2					**0**	**0**	**0**	**0**
CB1-9	44.6 ± 6.1		R, S, Dark		R, S, Dark	**0**	**3**	**0**	**3**
CB1-10	50.1 ± 4.9		Dark		R, S	**0**	**1**	**0**	**2**
CB1-11	72.1 ± 9.1	Deg	Deg			**4**	**4**	**0**	**0**
CB1-12	93.7 ± 7.3	R, O, Dark	R, O, Dark			**3**	**3**	**0**	**0**
CB1-13	119 ± 16					**0**	**0**	**0**	**0**
CB1-14	155 ± 19	S	O	R	Dark	**1**	**1**	**1**	**1**
CB1-15	186 ± 19		R	R, S	Dark	**0**	**1**	**2**	**1**
CB1-16	202 ± 25		R, Dark		Dark	**0**	**2**	**0**	**1**
CB1-17	212 ± 10		R, S	S		**0**	**2**	**1**	**0**
CB1-18	425 ± 46		Dark		Dark	**0**	**1**	**0**	**1**
CB1-19	477 ± 43	R				**1**	**0**	**0**	**0**
CB1-20	541 ± 76		Deg	S	R	**0**	**4**	**1**	**1**
CB1-21	665 ± 98	R				**1**	**0**	**0**	**0**
CB1-22	629 ± 57	R, O, Dark	R, O, Dark			**3**	**3**	**0**	**0**
CB1-23	1126 ± 120	R	R, Dark			**1**	**2**	**0**	**0**
CB1-24	1253 ± 173	R, O, Dark	R, O, Dark			**3**	**3**	**0**	**0**
CB1-25	3449 ± 346		R, Dark			**0**	**2**	**0**	**0**
CB1-26	6252 ± 262		R, Dark			**0**	**2**	**0**	**0**
CB1-27	9517 ± 607	R	R			**1**	**1**	**0**	**0**

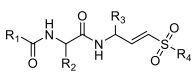
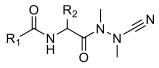
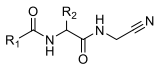
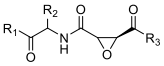
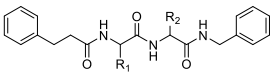
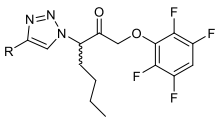
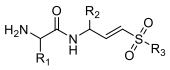
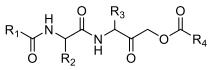
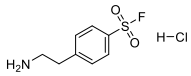
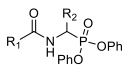
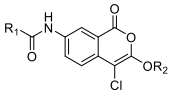
Název inhibitoru	Inhibice SmCB1 IC_{50} (nM)	Indukované fenotypy				Stupeň závažnosti fenotypu			
		10 µM		1 µM		10 µM		1 µM	
		1 d	2 d	1 d	2 d	1 d	2 d	1 d	2 d
CB1-28	9990 ± 280		Deg			0	4	0	0
CB1-29	$10^4 – 10^5$	R, S, Dark	Deg	R, S	R, S, Dark	3	4	2	3
CB1-30	$10^4 – 10^5$	R, S	R			2	1	0	0
CB1-31	$10^4 – 10^5$	R				1	0	0	0
CB1-32	n.i.		R, S		Dark	0	2	0	1
CB1-33	n.i.	S		S		1	0	1	0
CB1-34	n.i.		Dark			0	1	0	0

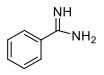
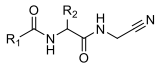
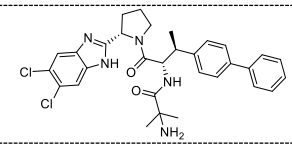
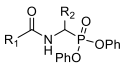
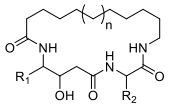
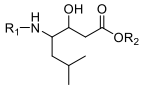
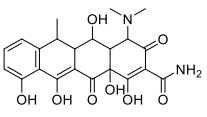
V rámci disertační práce bylo k identifikaci a validaci proteas *S. mansoni* jakožto molekulárních cílů testováno celkem 240 inhibitorů proteas (Tab. 3, str. 48-50). Inhibitory byly vybrány z knihoven chemických látek navržených pro inhibici lidských enzymů. Testované inhibitory byly specifické pro různé třídy a typy proteas. Cílem mojí práce bylo provézt komplexní genomický test se získanými inhibitory na živých schistosomulách, určit hlavní proteasy nezbytné pro přežití parazita a určit typy inhibitorů, které by mohly sloužit pro další vývoj inhibitorů k léčbě schistosomózy.

Jako proteasy kritické pro životaschopnost schistosomul a tím i potenciální molekulové cíle byly identifikovány trávicí proteasy: katepsiny B1, katepsin L, katepsin C a katepsin D a tegumentální proteasy prolyloligopeptidasa. Mírný fenotypový efekt byl sledován u dipeptidylpeptidasy IV a žádný u testovaných inhibitorů legumainu, metaloproteas a serinových proteas rodiny S1.

Tabulka 3: Přehled hlavních skupin inhibitorů testovaných chemickou genomikou. Rodina – zařazení proteas dle databáze MEROPS; Počet – počet testovaných inhibitorů; Účinnost = A: alespoň jeden ze skupiny inhibitorů se stejnou obecnou strukturou byl účinný v ex vivo testech (vykazoval změnu fenotypu); účinnost = N: žádný inhibitor se stejnou obecnou strukturou nebyl účinný v ex vivo (nevykazoval změnu fenotypu).

Rodina	Proteasa	Reaktivní skupina	Obecná struktura	Mechanismus	Počet	Účinnost
Cysteinové proteasy						
C1	CatB/CatL	azabenzoxazolát		kovalentní ireverzibilní	12	A

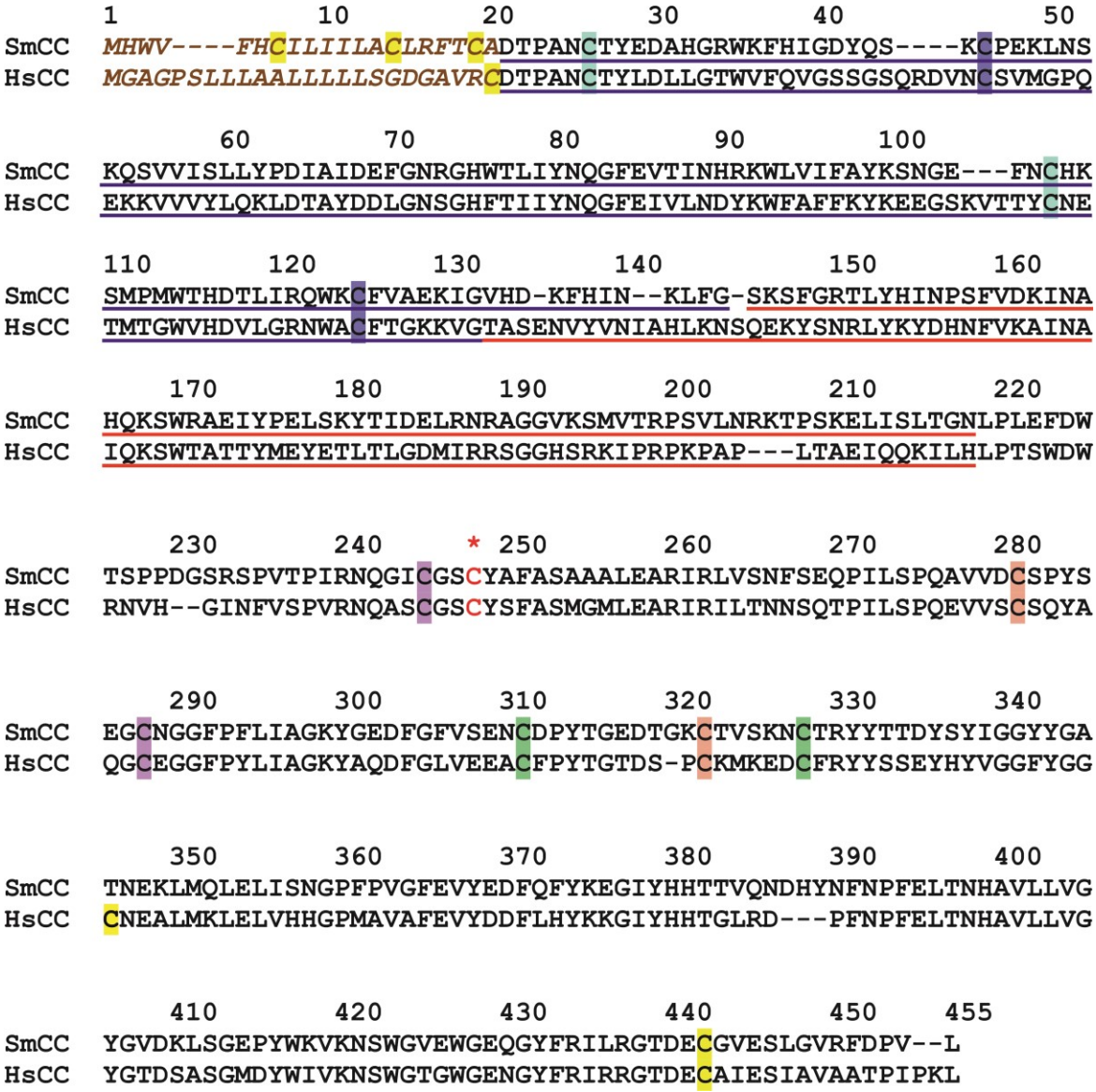
Rodina	Proteasa	Reaktivní skupina	Obecná struktura	Mechanismus	Počet	Účinnost
Cysteinové proteasy						
		vinylsulfony		kovalentní ireverzibilní	30	A
		azanitrily		kovalentní reverzibilní	23	A
		carbanitrily		kovalentní reverzibilní	40	A
		epoxidy		kovalentní ireverzibilní	4	A
		peptidy blokované na N-, C- konci; heterocykly a peptidomimetika		nekovalentní	21	A
C1	CatC	tetrafluorofenoxy arylmethylketony		kovalentní ireverzibilní	13	A
		vinylsulfony		kovalentní ireverzibilní	9	N
C13	legumain	acyloxymethylketony		kovalentní ireverzibilní	3	N
Serinové proteasy						
S1	SP1-5	sulfonyl fluorid		nekovalentní	1	N
		fosfonáty		kovalentní ireverzibilní	6	N
		cumariny		nekovalentní	3	N
		proteinové	Aprotinin	nekovalentní	1	N

Rodina	Proteasa	Reaktivní skupina	Obecná struktura	Mechanismus	Počet	Účinnost
Serinové proteasy						
		benzamidin		nekovalentní	1	N
S28	PrC (DPPIV)	nitrily		kovalentní reverzibilní	3	N
		dichlorobenzimid azolopyrrolidinam id		nekovalentní	1	A
		fosfonát		kovalentní ireverzibilní	1	N
S9	POP	aldehydy		reverzibilní; kovalentní	7	A
		halomethylketony		kovalentní ireverzibilní	2	A
Aspartátové proteasy						
A1	CatD	makrocyklické statiny		nekovalentní	39	A
		statiny		nekovalentní	5	A
Metaloproteasy						
M		nespecifické MP		nekovalentní	12	N
M10	MMP	tetracyklinové		nekovalentní	1	N

4.3.2. Katepsin C ze *S. mansoni* (SmCC)

Připravovaný rukopis č. 2 se zabývá charakterizací katepsin C ze *S. mansoni* (SmCC). Sekvence SmCC (Smp_019030) byla identifikována v databázi genomu *S. mansoni*

(Berriman et al. 2009; Protasio et al. 2012) analýzou programem BLASTp za použití sekvence lidského katepsinu C jako templátu. Teoretická molární hmotnost SmCC je 49 kDa a jeho sekvence obsahuje 455 aminokyselin. SmCC je z 44 % homologní s lidským katepsinem C (Obr. 19, str. 51) a patří do rodiny C1 cysteinových proteas. Pomocí programu SignalP byla predikována signální sekvence o délce 20 aminokyselinových zbytků (Obr. 19, str. 51). Dle homologie se sekvencí lidského katepsinu C, SmCC obsahuje N-terminální doplňkovou doménu a C-terminální katalytickou doménu obsahující katalytické zbytky Cys248 a His382. Mezi doplňkovou a katalytickou doménou se v sekvenci nachází aktivační peptid.



Obrázek 19: Porovnání sekvence SmCC a lidského katepsinu C (HsCC) (databáze Uniprot, kód: P53634). Signální sekvence byla predikována programem SignalP a je označena hnědou kurzívou, cysteinové zbytky tvořící disulfidový můstek jsou označeny stejnou barvou, modře je

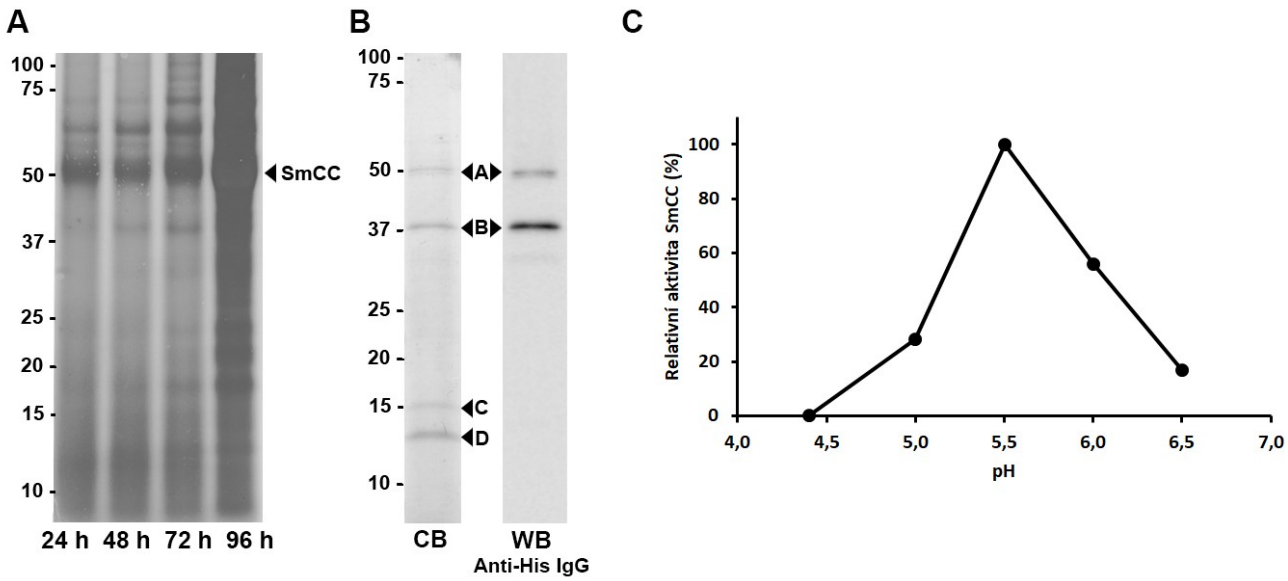
podtržená doplňková doména, červeně je podtržen aktivační peptid a aktivní cystein je znázorněn červenou hvězdičkou. N-konec aktivačního peptidu SmCC byl určen pro rekombinantní SmCC (viz kapitola 4.3.2.1.).

4.3.2.1. Příprava rekombinantního SmCC

Rekombinantní SmCC byl produkován expresí v prvokovi *L. tarentolae* dle firemního manuálu (Jena Bioscience) (1.9.2016). Tento expresní systém zaručuje správné sbalení a posttranslační modifikace proteinu. Úsek cDNA odpovídající zymogenu SmCC byl vložen do plasmidu pLEXSY-sat2 pro konstitutivní expresi ve čtecím rámci na N-konci se signální sekvencí LmSAP1, která zajišťuje sekreci rekombinantního produktu do média, a s histidinovou kotvou na C-konci, umožňující purifikaci pomocí chelatační chromatografie.

Časový průběh exprese byl analyzován ve vzorcích kultivačního média pomocí SDS-PAGE (Obr. 20A, str. 53). Teoretická molekulová hmotnost vypočtená ze sekvence proenzymu (zymogenu) SmCC je 50 kDa. Produkt o této molekulové hmotnosti je viditelný na SDS-PAGE již po jednom dni kultivace. Rekombinantní zymogen byl purifikován z expresního media po 48 h kultivace pomocí kombinace gelové a Ni^{2+} afinitní chelatační chromatografie. Preparát SmCC po purifikaci byl analyzován pomocí SDS-PAGE (Obr. 20B, str. 53) a detekcí na imunoblotu se specifickými protilátkami proti histidinové kotvě (Obr. 20B, str. 53). Purifikací byl získán preparát SmCC obsahující nejen proenzym (pás o molekulární hmotnosti 50 kDa), ale také pásy o nižší molární hmotnosti (37 kDa, 15 kDa a 13 kDa) představující fragmenty SmCC. Tyto pásy byly analyzovány pomocí hmotnostní spektrometrie a N-koncovým sekvenováním. Analýza potvrdila, že pás o molární hmotnosti 50 kDa je proenzym SmCC. Pásy fragmentů SmCC představují SmCC bez doplňkové domény (pás B na obrázku 20B (str. 53) s N-terminální sekvencí SKSFG-) a pásy doplňkové domény (pás C a D na obrázku 20B s N-term. sekvencí DTPAN-). Pásy A a B byly detekovány také pomocí protilátky proti histidinové kotvě, která je umístěna na C-konci SmCC.

U purifikovaného SmCC preparátu byla změřena aktivita pomocí kinetického aktivitního testu se specifickým substrátem pro katepsin C (Gly-Arg-AMC) a stanoven pH profil (Obr. 20C, str. 53). SmCC štěpil substrát mezi pH 4,5 až 6,5 s optimální aktivitou v pH 5,5.



Obrázek 20: A) Časový průběh exprese SmCC v expresním systému L. tarentolae při 26°C. Označený pás o 50 kDa odpovídá molekulové hmotnosti proenzymu SmCC. B) SDS elektroforéza a western blot přečištěného SmCC po gelové a afinintní chromatografii. Pásy A-D byly analyzovány pomocí hmotnostní spektrometrie, N-koncového sekvenování a anti-histagových protilátek. C) pH profil rekombinantní proteasy SmCC. Aktivita byla měřena v kinetickém testu s fluorogenním substrátem Gly-Arg-AMC. Zobrazeny jsou střední hodnoty vyjádřené v procentech. Směrodatná odchylka byla stanovena z měření v triplikátech.

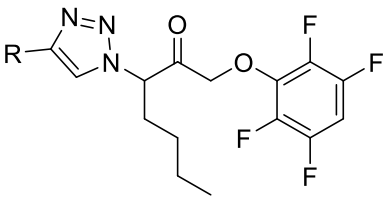
Z výsledků vyplývá, že se podařilo produkovat a purifikovat preparát obsahující směs intaktního proenzymu SmCC a proenzymu fragmentovaného pravděpodobně proteolytickým aparátem proteolytickým systémem *L. tarentolae* již v expresním médiu mezi doplňkovou doménou a aktivačním peptidem v pozici Gly144-S145 (Obr. 19, str. 51). Tento preparát SmCC je v malé míře aktivní. Pro další práci je nicméně nutné nalézt optimální aktivační protokol proenzymu SmCC za účelem získání dostatečného množství enzymu pro následné krystalizační pokusy.

4.3.2.2. TFPAMK inhibitory SmCC a jejich antischistosomální účinek

Inhibiční profil SmCC byl analyzován promocí tetrafluorofenoxyarylmethylketonových (TFPAMK) inhibitorů, které byly navrženy pro inhibici dipeptidyl peptidas z parazita *Plasmodia falcipari* (Li et al. 2012) a které poskytla laboratoř Dr. Bogya (Stanford University, USA). Nejúčinnější v testu s aktivitou SmCC v homogenátu dospělců byly inhibitory ML4118 a ML4118S (úplná inhibice při 10 nM koncentraci testovaného inhibitoru)

(Tab. 4, str. 54). Účinnost panelu inhibitorů byla dále testována na schistosomulách *S. mansoni* v kultivačním médiu a byly vyhodnoceny indukované změny fenotypů (Tab. 4, str. 54).

Tabulka 4: Inhibice aktivity SmCC a antischistosomální účinek syntetických TFPAMK inhibitorů. Inhibice SmCC byla určena pomocí kinetického aktivitního testu s homogenátem dospělých S. mansoni a fluorogenním substrátem Gly-Arg-AMC. V biologickém testu na živých schistosomulách byly popsány fenotypy vyvolané TFPAMK inhibitory (obecná kostra zobrazena v tabulce, jednotlivé ligandy v pozici R nejsou uvedeny) aplikovanými v 1, 5 a 10 μM koncentraci. Výsledné fenotypy pozorované ve třech časových bodech byly skórovány podle závažnosti účinku v rozmezí 0-4, přičemž stupeň 0 odpovídá fenotypu parazitů kultivovaných bez inhibitoru a stupněm 4 byl popsán nejzávažnější efekt.

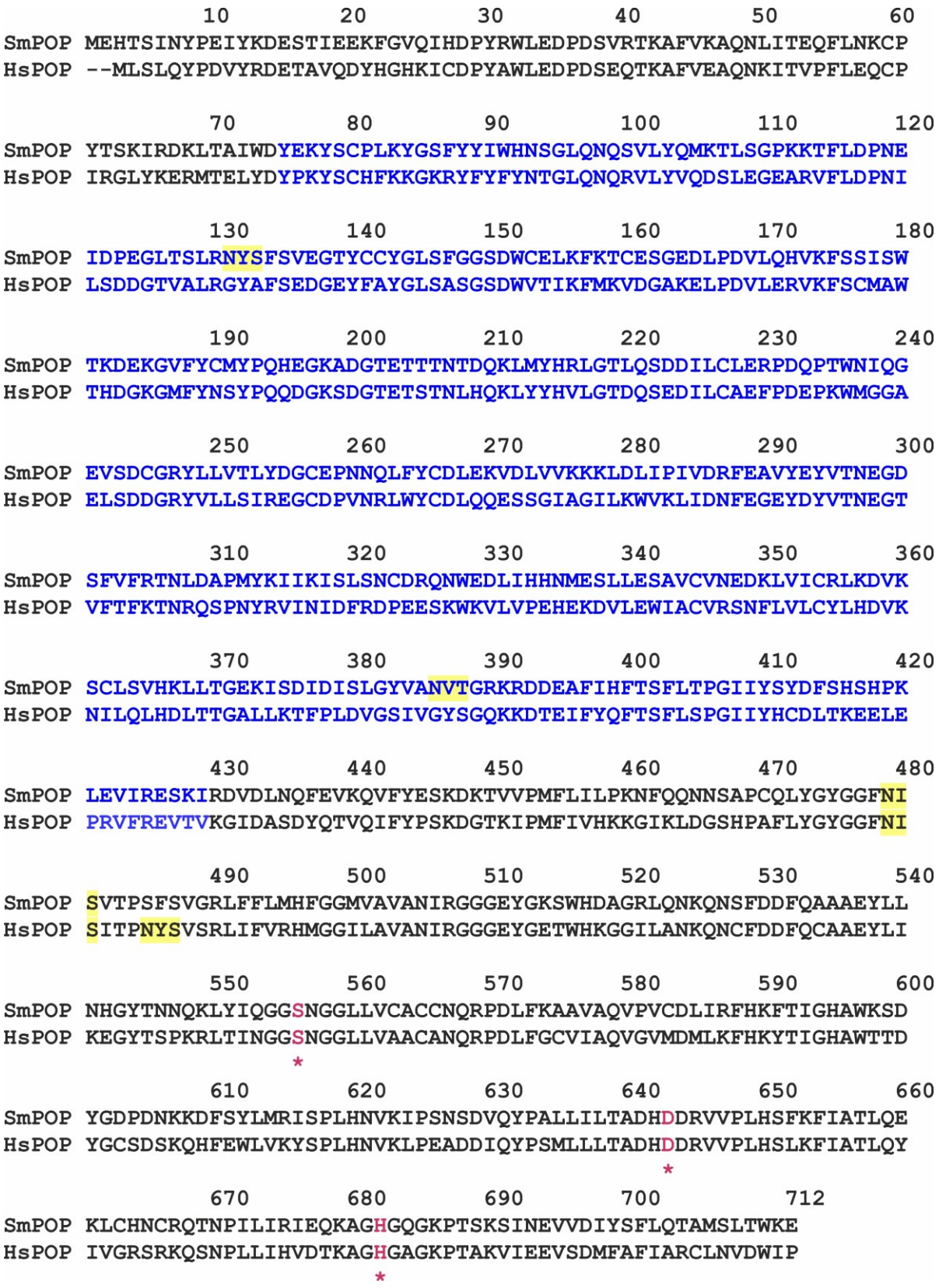


Název inhibitoru	Inhibice SmCC (%)			Stupeň závažnosti fenotypu								
				10 μM			5 μM			1 μM		
	1000 nM	100 nM	10 nM	1 d	2 d	3 d	1 d	2 d	3 d	1 d	2 d	3 d
ML4057	45	3	0									
ML4066	40	3	4									
ML4046A	92	43	0	4	4	4	4	4	4	1	3	4
ML4046B	8	0	0	4	4	4	0	0	0	0	0	0
ML4053	0	0	0	0	0	0				0	0	0
ML4068	71	13	0	1	4	4	0	0	0	0	0	1
HN3019	95	62	11	3	4	4				0	3	4
ML4161	95	68	17	0	2	4				0	3	4
ML4123	96	67	18				2	2	2	1	2	4
ML4118	99	97	92	3	4	4				0	3	3
ML4118S	100	100	100	4	4	4	4	4	4	4	4	4
ML4118R	95	66	26	1	4	4	0	0	1	0	3	2
ML4162	0	0	0	0	0	0				0	0	0
ML4163	9	0	0									
ML4154	52	4	0									
ML5004	0	0	0	2	4	4	2	4	4	0	1	0
ML6076	3	0	0	0	4	4	0	0	1	0	0	1
ML4091	85	41	2	0	0	4				0	2	2
ML4101	0	0	0									

Z výsledků vyplývá, že nejúčinnější inhibitory vykazovaly nejsilnější fenotypový efekt již v 1 μM koncentraci. Jako nejefektivnější byl identifikován inhibitor ML4118S, který byl velmi účinný v testech *ex vivo* i *in vitro.* Tento inhibitor bude podroben další analýze jakožto perspektivní templát pro vývoj nových antischistosomálních léčiv.

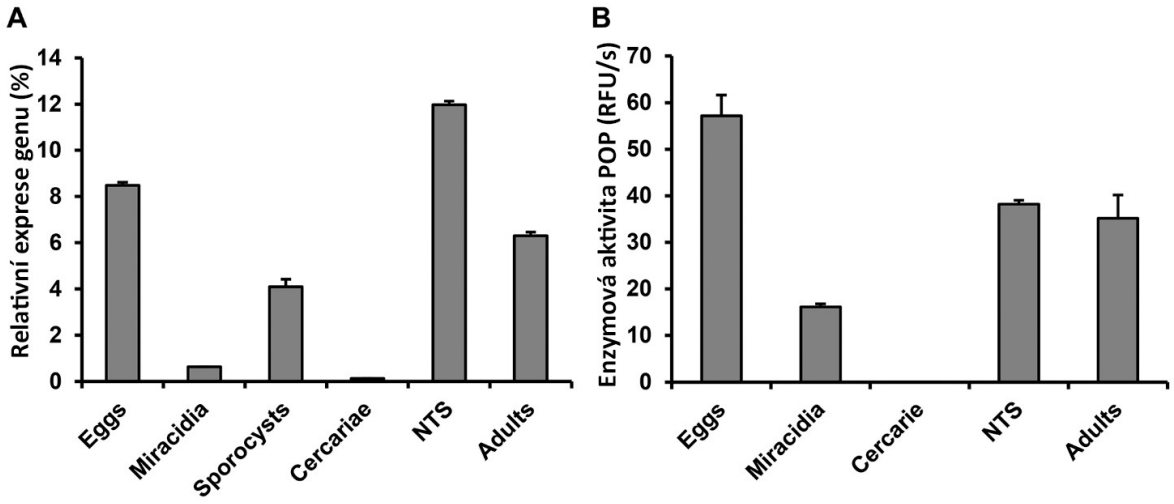
4.3.3. Prolyloligopeptidasa ze *S. mansoni* (SmPOP)

Publikace č. 4 se věnuje funkční biochemické charakterizaci SmPOP. Sekvence SmPOP (Smp_213240) byla identifikována v databázi genomu *S. mansoni* (Berriman et al. 2009; Protasio et al. 2012) analýzou programem BLASTp za použití sekvence lidské prolyloligopeptidasy jako templátu. Teoretická molární hmotnost SmPOP je 82 kDa a její sekvence obsahuje 712 aminokyselin. SmPOP je z 50 % homologní k lidské POP (Obr. 21, str. 56) a patří do rodiny S9 serinových proteas. Signální sekvence nebyla predikována. Dle homologie sekvencí, SmPOP obsahuje N-terminální cylindrickou doménu nazývanou β-propeler a katalytickou α/β hydrolasovou doménu, která obsahuje katalytickou triádu v pořadí Ser556, Asp643 a His682. Toto doménové složení je typické pro savčí POP a pro proteasy z rodiny S9 (Cunningham a O'Connor 1997).



Obrázek 21: Porovnání sekvence SmPOP a lidské prolyloligopeptidasy (HsPOP) (databáze Uniprot, kód: P48147). Modře je vyznačena specifická doména pro prolyloligopeptidasy tzv. „β-propeler," žlutě potenciální N-glykosylační místa a růžově katalytická triáda Ser, Asp, His.

RT-qPCR analýza exprese SmPOP v různých vývojových stádiích krevničky ukázala, že SmPOP je exprimovaná ve vejcích, dceřiných sporocystách, schistosomulách a dospělcích (a jen v malé míře v cerkáriích a miracidiích (Obr. 22A, str. 57). Enzymová aktivita SmPOP byla měřena v extraktech krevničky v kinetickém testu s fluorogenním substrátem Z-Gly-Pro-AMC, který je specifický pro prolyloligopeptidasy. Nejvyšší aktivita byla naměřena v homogenátech vajec, schistosomul a dospělců. Mírná aktivita byla změřena v homogenátu miracidií a v homogenátu cerkárií nebyla detekována žádná aktivita (Obr. 22B, str. 57).

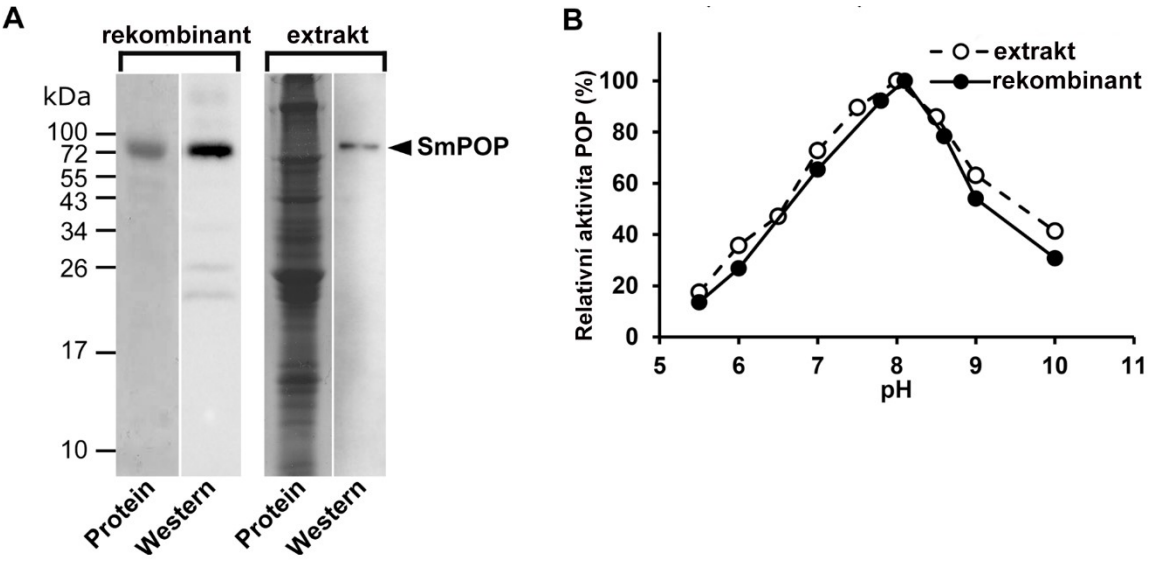


Obrázek 22: Aktivitní a transkripční profil SmPOP ve vývojových stádiích S. mansoni. A) Exprese SmPOP byla vyhodnocena pomocí RT-qPCR. Úrovně transkripce mRNA jsou uvedeny jako procentuální vyjádření vzhledem ke konstitutivně exprimované cytochromoxidase I ze S. mansoni. B) Aktivita SmPOP byla měřena v proteinových homogenátech vývojových stádií S. mansoni pomocí kinetické analýzy s fluorogenním substrátem Z-Gly-Pro-AMC v pH 8.0 a vztažena na obsah proteinů v homogenátu.

4.3.3.1. Příprava rekombinantní SmPOP

Rekombinantní proteasa SmPOP byla produkována v bakteriálním systému *E. coli* jako rozpustný a katalyticky aktivní enzym (Fajtová 2011) a purifikována kombinací chelatační a ionexové chromatografie. Purifikovaná SmPOP byla vizualizována na SDS elektroforéze jako jeden pás o velikosti 80 kDa. Polyklonální protilátky připravené imunizací králíků s SmPOP jako antigenem rozpoznávaly na imunoblotu jak antigen, tak zároveň i jeden pás v homogenátu z dospělých schistosom (Obr. 23A, str. 58). Molární hmotnosti rekombinantní i přirozené SmPOP (82 kDa) odpovídají teoretické molekulové hmotnosti vypočtené z aminokyselinové sekvence.

pH profil rekombinantní SmPOP byl stanoven pomocí kinetického testu s fluorogenním substrátem Z-Gly-Pro-AMC a porovnán s pH profilem nativní SmPOP v homogenátu z dospělých červů (Obr. 23B, str. 58). Oba enzymy štěpily substrát mezi pH 6,0 - 10,0 s optimální aktivitou v pH 8,0.



Obrázek 23: Porovnání nativní a rekombinantní SmPOP. A) Rekombinantní SmPOP exprimovaná v E. coli (dvě dráhy vlevo) a proteinový extrakt z dospělců S. mansoni (dvě dráhy vpravo) byly rozděleny na SDS elektroforéze, přeneseny na membránu a vizualizovány proteinovým barvením či imunodetekovány pomocí protilátek proti SmPOP. B) pH profil rekombinantní SmPOP a nativní SmPOP (v homogenátu dospělců S. mansoni). Aktivita byla měřena v kinetickém testu s fluorogenním substrátem Z-Gly-Pro-AMC. Aktivita je vyjádřena v procentech jednotek RFU/s normalizovaných na nejvyšší naměřenou hodnotu (100 %).

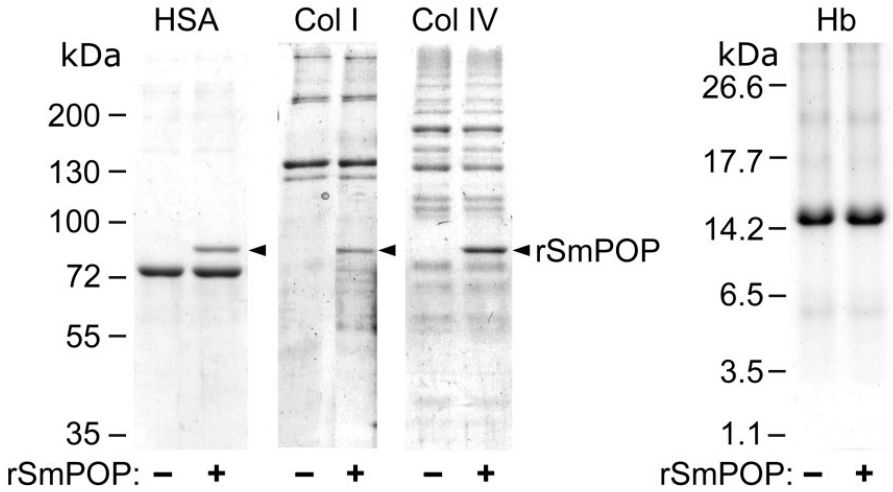
4.3.3.2. Substrátová specificita SmPOP

Prolyloligopeptidasy specificky štěpí peptidové substráty za prolinem. Pomocí panelu bioaktivních peptidů, které ve své sekvenci obsahují prolin, byla analyzována substrátová specificita SmPOP (Obr. 24 str. 59). Peptidy byly inkubovány s SmPOP, vzniklé fragmenty rozděleny pomocí HPLC a výsledná místa štěpení byla identifikována pomocí hmotnostní spektrometrie. Všechny substráty byly specificky štěpeny za prolinovým zbytkem s vyjímkou vazby Pro-Lys u substance P a Pro-Pro u bradykininu (Obr. 24, str. 59). Substrátová specificita SmPOP se podobá specificitě savčích prolyloligopeptidas, které štěpí vazby Pro-Xaa, kde Xaa je jakákoliv aminokyselina kromě prolinu. Stejně jako savčí prolyloligopeptidasy SmPOP neštěpí za předposledním prolinovým zbytkem na N-konci substrátu (Wilk 1983).

Peptid	Identifikovaná fragmentační místa
Angiotensin I	D R V Y I H **P** F H L
Angiotensin II	D R V Y I H **P** F
Bradykinin	R **P** **P** G F S **P** F R
LHRH	pE H W S Y G L R **P** G-amid
α-MSH	ac-S Y S M E H F R W G K **P** V-amid
Neurotensin	pE L Y E N K **P** R R **P** Y I L
Oxytocin	C Y I Q N C **P** L G-amid [Disulfid 1-6]
Substance P	R **P** K **P** Q Q F F G L M-amid
Vasopresin	C Y F Q N C **P** R G-amid [Disulfid 1-6]

Obrázek 24: SmPOP štěpí lidské peptidové hormony a neuropeptidy obsahující prolin. Peptidy byly inkubovány s rekombinantní SmPOP v pH 8,0 a místa štěpení (červené trojúhelníky) byly identifikovány pomocí hmotnostní spektrometrie. Proliny jsou vyznačeny tučně, disulfidové můstky v závorce.

Schopnost SmPOP štěpit makromolekulární proteiny hostitele byla testována se sadou lidských proteinů jako hemoglobin, sérový albumin a kolagen typu I a IV. Nebyla pozorována žádná fragmentace (Obr. 25, str. 59), což naznačuje, že SmPOP je oligopeptidasou štěpící pouze oligopeptidové, ale ne proteinové substráty.



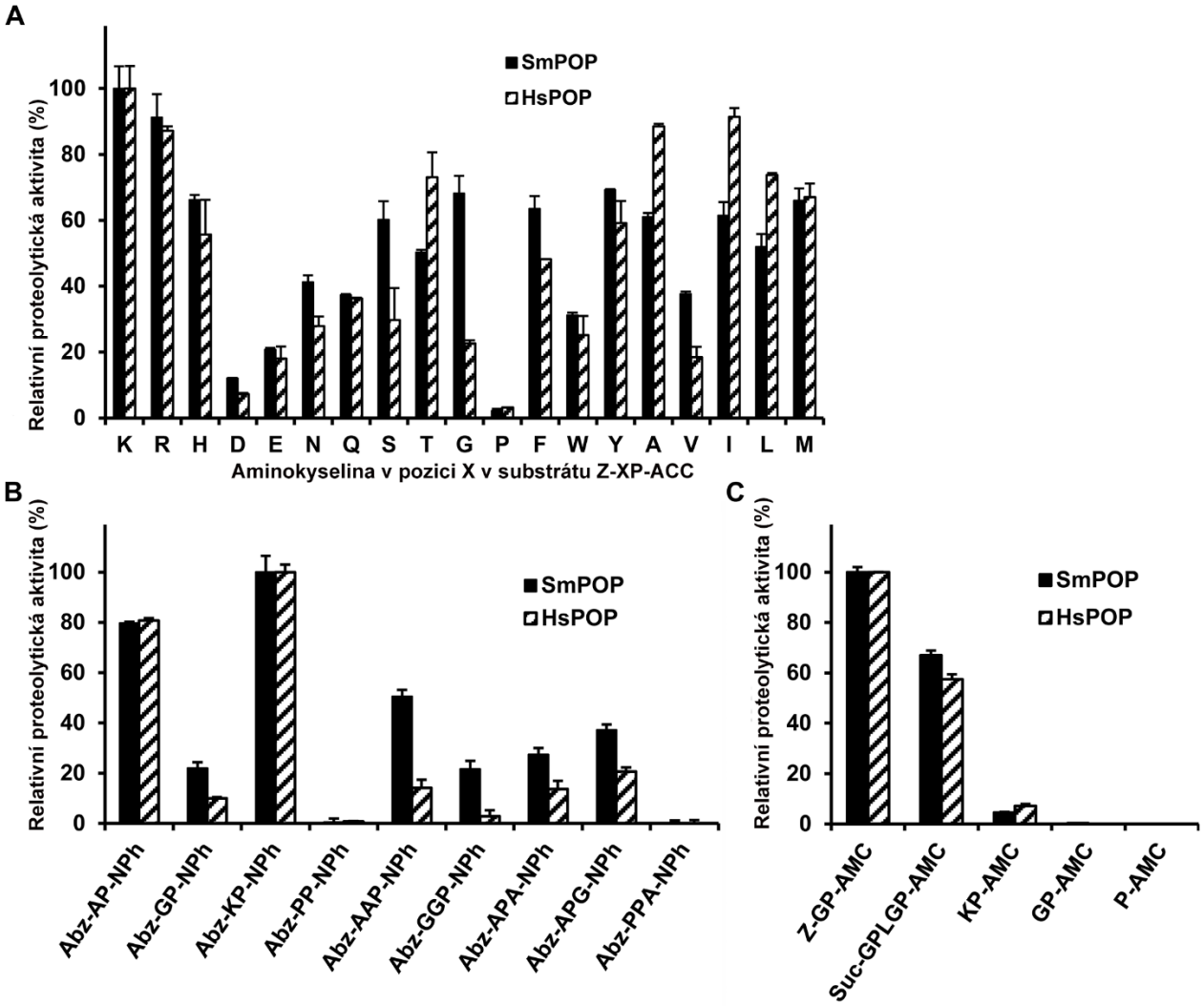
Obrázek 25: Rekombinantní SmPOP neštěpí makromolekulární proteinové substráty. Lidský sérový albumin (HSA), kolagen typu I a typu IV (Col I, Col IV) a hemoglobin (Hb) byly inkubovány 12 h v přítomnosti nebo bez rekombinantní SmPOP. Reakční směs byla

analyzována na SDS elektroforéze (HSA, Col I a Col IV) nebo na tricinové SDS elektroforéze (Hb) a proteiny byly *obarveny roztokem Coomassie Brilliant Blue G250.*

Substrátová specificita SmPOP v pozici P_2 byla studována pomocí fluorogenní substrátové knihovny (Obr. 26, str. 61). SmPOP preferovala substráty, které měly v pozici P_2 arginin nebo lysin, dále akceptovala substráty s hydrofobními, alifatickými a polárními aminokyselinami v pozici P_2. Nejméně preferované substráty byly s prolinem v pozici P_2.

Substrátová specificita byla dále studována pomocí peptidových FRET substrátů, které využívají jako donor fluorescenční skupinu aminobenzoyl (Abz) a jako akceptor nitrophenylalanin (NPh) a obsahují prolin v pozici P_1. Byla připravena sada substrátů s odlišnými aminokyselinovými zbytky v pozici P_2 (Abz-Ala-Pro-NPh, Abz-Gly-Pro-NPh, Abz-Lys-Pro-NPh, a Abz-Pro-Pro-NPh). Tyto substráty byly také prodlouženy do pozice P_3 (Abz-Ala-Ala-Pro-NPh and Abz-Gly-Gly-Pro-NPh) nebo pozice P_1' (Abz-Ala-Pro-Ala-NPh a Abz-Ala-Pro-Gly-NPh). Největší aktivita SmPOP byla naměřena se substráty Abz-Ala-Pro-NPh a Abz-Lys-Pro-NPh, zatímco substrát Abz-Pro-Pro-NPh nebyl hydrolyzován. Při prodloužení substrátu o jednu aminokyselinu do pozice P_3 a P_1' nedošlo ke zvýšení afinity substrátu k POP.

Nakonec byla testována schopnost rekombinantní SmPOP hydrolyzovat substráty s prolinem v pozici P_1, které jsou specifické pro jiné enzymy štěpící za prolinem jako: enzymy kolagenasového typu (Suc-Gly-Pro-Leu-Gly-Pro-AMC), dipeptidylaminopeptidasa II (Lys-Pro-AMC), dipeptidylaminopeptidasa IV (Gly-Pro-AMC) a prolylaminopeptidasa (Pro-AMC). Pouze Suc-Gly-Pro-Leu-Gly-Pro-AMC byl štěpen stejně účinně jako obecně používaný specifický substrát pro prolyloligopeptidasy Z-Gly-Pro-AMC. Naproti tomu hydrolýza substrátů s volným N-koncem byla velmi limitovaná (Lys-Pro-AMC) anebo neprobíhala vůbec (Gly-Pro-AMC a Pro-AMC).



Obrázek 26: Substrátová specificita rekombinantní SmPOP. Aktivita SmPOP byla měřena pomocí sady syntetických substrátových knihoven: A) fluorogenní substráty, které obsahovaly prolin v pozici P_1, se strukturou Z-X-Pro-ACC, kde X je jedna z proteinogenních aminokyselin v pozici P_2, ACC je fluorofor 7-amino-4-karbamoylmethylkumarin; B) peptidové FRET substráty s prolinem v pozici P_1 a aminokyselinovými zbytky Ala, Gly, Pro, Lys nebo Gly v pozici P_2, které byly také prodlouženy o jednu aminokyselinu do pozice P_3 nebo P_1'; C) fluorogenní substráty s prolinem v pozici P_1, které jsou používány k měření aktivit následujících peptidas: kolagenasy (Suc-GPLGP-AMC), dipeptidylaminopeptidasy II (KP-AMC), dipeptidylaminopeptidasy IV (GP-AMC) a prolylaminopeptidasy (P-AMC). Hydrolýza substrátů byla měřena v kinetickém testu v pH 8,0 s rekombinantní SmPOP nebo lidskou POP (HsPOP).

Výsledky prokázaly, že SmPOP je oligopeptidasa hydrolyzující oligopeptidové substráty v endopeptidolytickém módu na karboxylovém konci prolinového zbytku, která neštěpí

makromolekulární proteinové substráty. Z biochemických a strukturních analýz vyplývá, že SmPOP a savčí POP mají téměř stejnou substrátovou specificitu, což naznačuje silné evoluční zachování funkce a struktury.

4.3.3.3. Inhibiční specificita SmPOP

Inhibiční specificita SmPOP byla analyzovaná pomocí panelu malých inhibitorů specifických pro různé třídy a typy proteas (Tab. 5, str. 62-63). Aktivita SmPOP byla zcela inhibovaná selektivními inhibitory prolyloligopeptidas s chloromethyl ketonovou (CMK) nebo aldehydovou (CHO) reakční skupinou (Z-Ala-Pro-CMK a Z-Pro-Pro-CHO), dále pak obecným inhibitorem serinových proteas diisopropyl fluorofosfátem. Pouze částečná inhibice byla pozorována u AEBSF (4-(2-aminoethyl)benzenesulfonylfluoridhydrochlorid), PMSF (fenylmethylsulfonylfluorid), TLCK (Nα-tosyl-L-lysin chloromethyl keton), TPCK (N-p-tosyl-L-fenylalanin chloromethyl keton) a 3,4-dichloroisocoumarin, všechny tyto inhibitory jsou specifické pro serinové proteasy chymotrypsinové rodiny. Aktivita SmPOP nebyla ovlivněna proteinovými inhibitory STI (soybean trypsin inhibitor) a BPTI (bovine pancreatic trypsin inhibitor) ani inhibitory cysteinových, aspartátových nebo metaloproteas. Celkový inhibiční profil pro SmPOP odpovídá profilům popsaným pro savčí prolyloligopeptidasy.

Tabulka 5: Inhibice SmPOP inhibitory proteas. Hodnota inhibice byla určena pomocí kinetického testu s rekombinantní SmPOP a fluorogenním substrátem Z-Gly-Pro-AMC v pH 8,0; AP – aspartátové proteasy, CP – cysteinové proteasy, MP – metaloproteasy, SP – serinové proteasy.

Inhibitor	Cílová proteasa	Koncentrace (μM)	Inhibice (%)
AEBSF	SP	1000	12,0±3,1
PMSF	SP	1000	47,6±1,6
Benzamidin	SP (trypsinového typu)	10	3,7±1,1
TLCK	SP (trypsinového typu)	1	38,3±1,2
TPCK	SP (chymotrypsinového typu)	1	67,2±6,2
3, 4-dichloroisocoumarin	SP	100	77,3±0,6
BPTI	SP	50	1,4±1,1
STI	SP	10	12,3±3,2
Diisopropyl fluorofosfát	SP	100	100±1
Leupeptin	SP, CP	20	2,3±1,2

Inhibitor	Cílová proteasa	Koncentrace (μM)	Inhibice (%)
Antipain	SP, CP	20	32,4±1,4
E-64	CP	10	6,5±6,1
Pepstatin A	AP	1	7,3±3,5
EDTA	MP	1000	3,8±2,3
Bestatin	MP (leucinaminopeptidasa)	1	2,3±2,1
Z-Ala-Pro-CMK	SP (prolyloligopeptidasa)	1	100±3
Z-Pro-Pro-CHO	SP (prolyloligopeptidasa)	1	100±1
Z-Pro-Pro-OH	SP (prolyloligopeptidasa)	100	37,6±2,1
Z-Pro-OH	SP (prolidasa)	100	41,1±1,8

Detailnější inhibiční profil SmPOP byl analyzován pomocí panelu syntetických peptidových inhibitorů se strukturou Z-Xaa-Pro-CHO/CMK, které obsahovaly aldehydovou (CHO) nebo chloromethyl ketonovou (CMK) reaktivní skupinu (Tab. 5, str. 62-63). Aminokyseliny v pozici P_2 (Xaa) byly vybrány na základě získané substrátové specificity (Obr. 26, str. 61). Hodnoty IC_{50} aldehydových derivátů dosahovaly mikromolárních hodnot (1,3 - 6,1 μM). Inhibiční specificita v pozici P_2 odpovídala nalezené substrátové specificitě SmPOP: nejnižší hodnoty IC_{50} byly naměřeny pro inhibitory, které obsahují bazické aminokyseliny v pozici P_2. Zavedením ireversibilní chloromethyl ketonové reaktivní skupiny do inhibitorů došlo k poklesu IC_{50} hodnot o tři řády (IC_{50} od 2,9 nM do 3,2 nM).

Dále byla testována účinnost inhibitorů vyvinutých proti lidské POP: Y-29794 oxalát (Nakajima et al. 1992), SUAM 14746 (Lawandi et al. 2010) a Z-Pro-Pro-CHO (Yoshimoto et al. 1985). Zatímco SUAM 14746 inhiboval lidskou i schistosomální POP stejně efektivně (IC_{50} = 83 nM pro HsPOP a 92 nM pro SmPOP), hodnoty pro inhibici SmPOP inhibitory Y-29794 oxalát a Z-Pro-Pro-CHO byly o řád vyšší než pro lidskou POP. (Hodnoty IC_{50} = 8,6 μM (HsPOP) a 0,49 μM (SmPOP), pro Y-29794 oxalát, a 0,16 μM (HsPOP) a 0,01 μM (SmPOP) pro Z-Pro-Pro-CHO. Inhibiční specificita kopírovala substrátovou specificitu POP s výjimkou Pro zbytku v pozici P_2: Z-Pro-Pro-CHO byl účinným inhibitor, ale Z-Pro-Pro-ACC špatný substrát. Stejná specificita byla pozorována i u lidské POP.

4.3.3.4. Antischistosomální účinek inhibitorů SmPOP

Účinnost panelu SmPOP inhibitorů byla dále testována na schistosomulách *S. mansoni* v kultivačním médiu vyhodnocením indukovaných změn fenotypů (Tab. 6, str. 64). Výsledky

ukázaly, že CHO inhibitor Z-Lys-Pro-CHO měl velmi slabý efekt, inhibitory Z-Gly-Pro-CHO, Z-Tyr-Pro-CHO, Z-Arg-Pro-CHO a CMK inhibitor Z-Arg-Pro-CMK vykazovaly signifikantní změnu fenotypu. Nejvýraznější efekt byl indukován inhibitory Z-Ala-Pro-CHO a Z-Ala-Pro-CMK. Komerční inhibitory lidské POP Y-29794 a SUAM 14746 indukovaly mírnou nebo žádnou změnu fenotypu.

Tabulka 6: Inhibice aktivity SmPOP a antischistosomální účinek syntetických inhibitorů SmPOP. Hodnota IC_{50} byla určena pomocí kinetického testu s rekombinantní SmPOP a fluorogenním substrátem Z-Gly-Pro-AMC v pH 8,0. V biologickém testu na živých schistosomulách byly sledovány změny fenotypu vyvolané inkubaci s inhibitory, testovanými v koncentracích 10 a 1 µM. Výsledné fenotypy pozorované ve třech časech byly zařazeny podle závažnosti účinku do tříd 0-4, přičemž stupeň 0 odpovídá fenotypu parazitů kultivovaných bez inhibitoru a stupněm 4 byl popsán nejzávažnější efekt; [1]SUAM-14746 – komerční inhibitor lidské prolyloligopeptidasy (Sigma-Adrich);[2]Y-29794 – komerční inhibitor lidské prolyloligopeptidasy (Santa Cruz Biotechnology).

Inhibice SmPOP		Stupeň závažnosti fenotypu					
		10 µM			1 µM		
	IC_{50} (µM)	1 d	2 d	3 d	1 d	2 d	3 d
Y-29794[1]	8.6 ± 0.4	2	4	4	1	2	2
SUAM-14746[2]	0.092 ± 0.005	1	0	0	0	0	0
Z-Pro-Pro-CHO	0.16 ± 0.03	1	0	0	0	0	0
Z-Ala-Pro-CHO	3.1 ± 0.2	1	1	4	0	1	2
Z-Gly-Pro-CHO	6.1 ± 0.4	2	2	4	1	1	2
Z-Tyr-Pro-CHO	4.4 ± 0.4	2	2	4	1	1	2
Z-Arg-Pro-CHO	1.3 ± 0.3	2	2	4	1	2	2
Z-Lys-Pro-CHO	3.0 ± 0.6	4	4	4	1	1	4
Z-Ala-Pro-CMK	0.0032 ± 0.0004	2	2	4	1	1	2
Z-Arg-Pro-CMK	0.0029 ± 0.0001	0	1	2	0	1	2

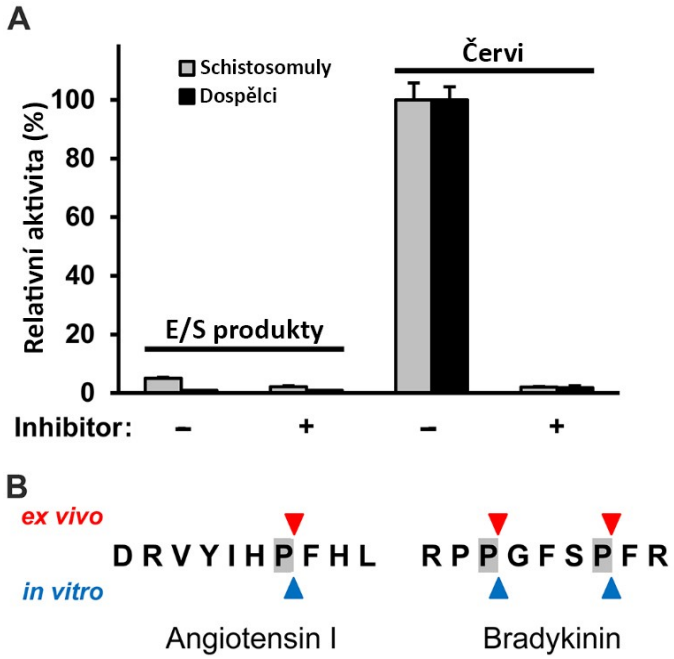
4.3.3.5. SmPOP v živých parazitech štěpí bioaktivní peptidy hostitele

V dalším kroku bylo zkoumáno, zda-li může SmPOP interagovat s peptidovými substráty v prostředí obklopujícím parazita. Schistosomuly nebo dospělci *S. mansoni* byly inkubovány v kultivačním mediu v mikrotitrační destičce s fluorogenním substrátem Z-Gly-Pro-AMC. Štěpení substrátu bylo detekováno pomocí fluorescenční čtečky (Obr. 27, str. 65). Aktivita SmPOP nebyla detekována, pokud byly paraziti současně se substrátem kultivovány i se specifickým SmPOP inhibitorem Z-Ala-Pro-CMK. V ES produktech odebraných po

dvoudenní kultivaci parazitů nebyla zjištěna žádná významná aktivita SmPOP, což ukazuje, že SmPOP není vylučována parazitem do kultivačního média.

V dalším kroku byly do kultivačního média s dospělými parazity přidány peptidové hormony angiotensin I a bradykinin. Oba hormony byly štěpeny a fragmentační místa identifikovaná pomocí hmotnostní spektrometrie byla lokalizována za zbytky prolinu. Fragmentace byla inhibována v přítomnosti specifického POP inhibitoru Z-Ala-pro-CMK. Zjištěná místa štěpení v sekvencích peptidů byla shodná s fragmentačními místy získanými inkubací *in vitro* s rekombinantní SmPOP.

Závěrem lze konstatovat, že i když není proteasa SmPOP parazitem sekretován, může interagovat s fyziologicky relevantními hostitelskými peptidy v prostředí.



Obrázek 27: Enzymová aktivita SmPOP v živých krevničkách. A) SmPOP aktivita v ES produktech a se živými schistosomulami nebo dospělými krevničkami kultivovanými v médiu byla měřena pomocí fluorogenního substrátu Z-Gly-Pro-AMC. Inhibitor Z-Gly-Pro-CMK byl přidán v kontrolních pokusech do média, aby specificky blokoval aktivitu SmPOP. B) Peptidové hormony angiotensin a bradykinin byly inkubovány v kultivačním médiu společně s živými dospělými krevničkami (ex vivo) nebo v reakční směsi s rekombinantní SmPOP (in vitro). Médium a reakční směs byly analyzovány pomocí hmotnostní spektrometrie a identifikovaná fragmentační místa byla označena červenými trojúhelníčky.

5. Diskuse

Schistosomóza je jednou z opomíjených tropických parazitárních onemocnění, která postihuje přes 200 milionů lidí a riziko infekce se týká 750 milionů lidí v 78 zemích spadajících do tropických a subtropických oblastí světa. Výslednou bilancí je schistosomóza z parazitárních infekcí na druhém místě za malárií a představuje jednoznačně globální zdravotní problém. Toto chronické parazitární onemocnění je způsobené krevničkami rodu *Schistosoma*.

Proteasy jsou nezbytné pro přežití krevniček v těle hostitele (Kasny et al. 2009). Jsou důležité pro invazi do hostitele, migraci, trávení, reprodukci a modulování hostitelských fyziologických funkcí a imunitní odpovědi. Proto jsou proteasy stále častěji studovány jako cílové molekuly pro nová chemoterapeutika a vakcíny proti schistosomóze. Dosavadní výzkum proteas *S. mansoni* se orientoval převážně na funkční charakterizaci jednotlivých proteas nebo anotaci jejich sekvencí v rámci genomových či transkriptomických studií. Cílem disertační práce bylo jednak detailně analyzovat složení proteolytického systému sekretovaného parazitem, který se významně podílí na interakci krevničky s lidským hostitelem, a jednak vyhledat a funkčně charakterizovat cílové molekuly pro vývoj antischistosomálních strategií a identifikovat jejich účinné inhibitory představujících potenciální léčiva proti schisostosomóze.

Mapování proteolytických aktivit sekretovaných krevničkou při interakci s lidským hostitelem

V první části disertační práce (**publikace č. 1-3**) byly hlavní typy enzymatických aktivit proteas sekretovaných krevničkou mapovány a klasifikovány pomocí moderních funkčně-proteomických metod. Charakterizována byla substrátová specificita nalezených serinových proteas (SP) rodiny S1 a nejvíce zastoupená proteasa SmSP2 byla podrobně biochemicky charakterizována.

V publikaci č. 1 (Dvořák et al. 2016) Byly připraveny exkrečně-sekreční (ES) produkty vývojových stádií *S. mansoni*, které žijí v lidském hostiteli, konkrétně dospělých schistosom, schistosomul (juvenilní schistosomy) a vajec. Tyto ES produkty, které byly připraveny za fyziologických podmínek, obsahují proteasy, které působí na rozhraní parazit-hostitel a potenciálně mohou ovlivňovat homeostázu hostitele. Aktivity sekretovaných proteas v ES produktech byly mapovány pomocí FRET substrátů a také nové multiplexové metody. Tato metoda byla doposud úspěšně použita jednak k identifikaci substrátových specifit

jednotlivých rekombinantních proteas (např. lidského katepsinu K (Sharma et al. 2015) a serinové proteasy z *Mycobacteria tuberculosis (Small et al. 2013)*), jednak ke komplexním analýzám proteolytických systémů (např. trávicích proteas z humra *Homarus americanus* (Bibo-Verdugo et al. 2016) a ES produktů cerkárií *S. mansoni* (O'Donoghue et al. 2012)).

Tímto způsobem bylo v ES produktech analyzovaných ve vývojových stádií krevničky nalezena sada proteolytických aktivit. Nejjednodušší profil byl detekován v ES produktech schisotosomul, kde převládá pravděpodobně jediná serinová proteasa se specifitou v pozicích P_1-P_1' pro bazické aminokyseliny. Profil je zcela odlišný od substrátové specificity nalezené v ES produktech cerkárií, stádia, ze kterého se schistosomuly vyvíjí. U cerkárií v ES produktech dominuje cerkáriová elastasa se substrátovou specificitou chymotrypsinového typu (specificita pro Phe a Tyr v pozici P_1 a Pro v pozici P_2). Naproti tomu v ES produktech u dospělých parazitů a vajec byly identifikovány komplexní profily, které se lišily v zastoupení tříd cysteinových a serinových proteas a metaloproteas, což naznačuje rozdílnou jejich rozdílnou důležitost v průběhu vývoje parazita. U všech vývojových stádií byla nalezena významná aktivita serinových proteas, a to především trypsinového typu (tj. aktivity štěpící za bazickými aminokyselinami Arg či Lys).

Vzhledem k významnému podílu serinových proteas rodiny S1 v ES produktech krevničky, se **publikace č. 2 (Horn at al. 2014)** orientovala na genomickou analýzu a klasifikaci proteas této rodiny. Převážná většina publikací o proteasách rodiny S1 se u krevniček zabývá cerkáriovou elastasou (Ingram et al. 2012; Salter et al. 2000), enzymu, který usnadňuje larvám krevničky pronikat kůží do těla hostitele. Z ostatní proteas rodiny S1 krevniček byla částečně charakterizována jen serinová proteasa 1 (SP1) (Cocude et al. 1999). Dále byla v proteinovém homogenátu dospělé krevničky identifikována aktivita serinových proteas kalikreinového typu a tato proteasa byla izolována a pojmenována SK1 (Carvalho et al. 1998). Tento enzym štěpí kalikreinové substráty a procesuje kininogen na aktivní bradykinin jenž následně vyvolal silnou vazodilataci a snížil arteriální krevní tlak u experimentálních krys (Carvalho et al. 1998). Obě proteasy SK1 a SP1 pravděpodobně hrají roli při regulaci vaskulárních funkcí hostitele. Další studie se věnovala aktivitě serinových proteas v extraktech vajec *S. mansoni,* kde vykazovala významnou fibrinolytickou aktivitu (Doenhoff et al. 2003).

V genomu krevničky střevní bylo nalezeno pět genů pro serinové proteasy rodiny S1, označených SmSP1-5 (**publikace č. 2**). Analýza jejich proteinových sekvencí ukázala, že proteasy SmSP1-4 jsou proteasy trypsinového typu a SmSP5 je proteasa chymotrypsinového typu. U SmSP1-4 je substrátová specificita podmíněna Asp v podmístě S_1, který určuje štěpení

substrátů v pozice P_1 za Arg, Lys (Huber a Bode, 1979), oproti tomu u SmSP5 se v podmístě S_1 nachází Gly. Díky této malé nepolární aminokyselině vykazuje SmSP5 pravděpodobně substrátovou specificitu podobnou proteasám chymotrypsinového typu.
Aktivity SmSP1-5 byly detekovány v homogenátech schistosomul, dospělců a vajec, pomocí kinetického měření se sadou specifických substrátů a byla testována jejich citlivost k inhibitorům selektivním pro proteasy rodiny S1. V kinetických testech převažovala trypsinová aktivita nad chymotrypsinovou, což odpovídá teoretickým specifitám odvozeným od charakteru S_1 vazebného místa. Převažující trypsinová aktivita byla detekována i v ES produktech, což znamená, že SmSP jsou sekretovány ve své aktivní formě z těla parazita a mohou hrát klíčovou roli v interakcích mezi parazitem a hostitelem.

Analýza exprese mRNA SmSP prokázala, že ve všech vývojových stádiích parazitujících u lidského hostitele je v nejvyšší míře exprimována proteasa SmSP2, a proto ji byla věnována další studie. V **publikaci č. 3 (Leontovyč et al. 2018**) byla SmSP2 biochemicky charakterizována a byla navržena její biologická role. SmSP2 byla popsána jako multidoménový enzym, vzdáleně připomínající doménovou strukturu serinových proteas koagulační kaskády. Podle charakteru S_1 vazebného podmísla byla SmSP2 klasifikována jako proteasa trypsinového typu. To bylo potvrzeno analýzou specificity pomocí substrátových knihoven, která ukázala preference pro bazické aminokyseliny Arg, Lys v pozici P_1. SmSP2 byla imunolokalizována na povrchu (tegumentu) krevničky, odkud je sekretovaná parazitem do média, kde může štěpit proteinové substráty hostitele. Bylo zjištěno, že SmSP2 štěpí pouze určité proteiny hostitele např. kininogen, fibrinogen a plasminogen, což naznačuje její roli v modulaci hemostázy hostitele.

Identifikace a charakterizace proteas krevničky jako cílových molekul pro potenciální antischistosomální léčiva

Druhá část disertační práce se zabývala identifikací povrchových a trávicích proteas krevničky *S. mansoni* jako cílových molekul pro vývoj nových léčiv proti schistosomóze. Chemická genetika používá ke studiu biologických procesů malé specifické molekuly, které jsou schopny modulovat funkce různých genových produktů (nejčastěji proteinů, ale také např. DNA nebo RNA); nejčastěji je sledován vliv těchto látek na fenotyp studovaného organismu.

V disertační práci byl studován vliv inhibitorů proteas na životaschopnost schistosomul v kultivačním médiu. Celkem bylo testováno 240 inhibitorů lidských homologů schistosomálních proteas, konkrétně se jednalo o inhibitory aspartátových proteas rodiny

pepsinu (katepsinu D), cysteinových proteas rodiny papainu (katepsinů B/L a C) a rodiny legumainu, dále serinových proteas (trypsinového typu, prolyloligopeptidasy a dipeptidylpeptidasy IV) a metaloproteas. Jako kritické byly identifikovány inhibitory trávicích proteas typu katepsinů B, L, C a D a tegumentálních proteas typu prolyloligopeptidasy. Mírná změna fenotypu byla nalezena po inkubaci s inhibitorem dipeptidylpeptidasy IV a žádný efekt neměly inhibitory legumainu, metaloproteas a serinových proteas rodiny S1.

Katepsin B1 ze *S. mansoni* (SmCB1) byl již dříve validován jako cílová molekula pro léčbu schistosomózy na myším modelu (Abdulla et al. 2007). Vinylsulfonový inhibitor K11777 způsobil u nakažených myší podstatný pokles počtu dospělých červů a produkce vajíček. V krevničkách, které byly izolovány z infikovaných myší, byla identifikována SmCB1 jako hlavní proteasa inhibovaná K11777. Tento výsledek následně podpořila porovnání derivátů K11777 testovaných *in vitro* s rekombinantním enzymem a *ex vivo* s živými krevničkami v kultivačním médiu (Jilkova et al. 2011); prokázaná byl korelace mezi inhibičními konstantami a vyvoláním poškozujícího fenotypu u živých krevniček. Na tuto práci navazuje připravovaný **rukopis č. 1 (Jílková et al. 2018),** ve které byla vyvinuta a testována sada druhé generace vinylsulfonových inhibitorů SmCB1. Získané inhibitory, které jsou dosud nejúčinnějšími vyvinutými inhibitory SmCB1, vykazují subnanomolárních inhibičních parametrů *in vitro*.

Další část disertační práce se věnuje funkční charakterizaci dvou proteas, které byly identifikovány jako potenciální cílové molekuly: trávicí proteasa katepsin C ze *S. mansoni* (SmCC) (připravovaný **rukopis č. 2 - Fajtová et al. 2018**) a tegumentální prolyloligopeptidasa ze *S. mansoni* (SmPOP) (**publikace č. 4 – Fajtová et al 2015**).

SmCC byl produkován rekombinantní expresí v prvokovi *L. tarentolae*. Výhodou tohoto expresního systému je především zachování řady posttranslačních modifikací eukaryotických proteinů. Tento expresní systém byl již úspěšně použit pro lysozomální proteiny (Breitling et al. 2002; Chen et al. 1997). Rekombinantní SmCC byl z expresního média získán ve formě neaktivního zymogenu a v malé míře i jako aktivní enzym, pravděpodobně aktivovaný proteolytickým aparátem hostitelských buněk *L. tarentolae*. Tento aktivní preparát byl dostačující pro základní charakterizaci biochemickou SmCC. V další fázi výzkumu bude nutné vyvinout aktivační protokol pro řízenou trans-aktivaci jinými proteasami (Dahl et al. 2001), např. pomocí dalších trávicích proteas krevničky, které se mohou podílet na aktivaci SmCC *in vivo*. Inhibiční profil SmCC byl testován se sadou

tetrafluorofenoxarylmethylketonových inhibitorů, které byly navrženy proti dipeptidylaminopeptidase I (DPAP I), orthologu SmCC z *Plasmodia falcipari*, který je původcem malárie (Deu et al. 2010). Podobně jako DPAP I byl SmCC inhibován *in vitro* nejúčinnějšími inhibitory v nanomolární oblasti koncentrací. Tyto inhibitory vykazovaly silný antischistosomální účinek ve fenotypovém testu na živých schistosomulách, přičemž nejefektivnější inhibitor ML4118S byl cca 1000x účinnější než praziquantel, v současnosti jediný používaný lék proti schistosomóze. ML4118S částečně inhibuje i katepsin B1 a L3 (nepublikovaná data), vysoká antischistosomální účinnost inhibitoru může být daná křížovou reaktivitou proti dalším trávicím proteasám, které vykazují synergický efekt.

V publikaci č. 4 (Fajtová et al 2018) byla identifikována a funkčně charakterizována prolyloligopeptidasa ze *S. mansoni* (SmPOP), serinová proteasa rodiny S9. SmPOP vykazuje 51 % identity se sekvencí lidské prolyloligopeptidasy. Homologní trojrozměrný model SmPOP prokázal shodnou doménovou prostorovou architekturu s lidskou POP, složenou z katalytické α/β hydrolasové domény a cylindrické domény nazývané β-propeler, která reguluje aktivní místo a zapřičiňuje oligopeptidasovou aktivitu enzymu (Fulop et al.1998).

Rekombinantní SmPOP byla produkována v expresním systému *E. coli.* Analýza substrátové a inhibiční specificity prokázala, že SmPOP podobně jako savčí POP (Polgar 2002) štěpí oligopeptidové substráty v endopeptidasovém módu za zbytkem Pro. SmPOP preferuje v pozici P_2 bazické aminokyseliny před hydrofobními a alifatickými, naproti tomu substráty s Pro v pozici P_2 nebyly štěpeny. SmPOP nebyla inhibována inhibitory S1 rodiny jako je AEBSF, benzamidin a BPTI. Tyto výsledky také odpovídají inhibiční specificitě pro savčí POP a POP z parazita *Trypanosoma cruzi* (nazvané Tc80) (Sharma and Ortwerth 1994; Bastos et al. 2010). Inhibiční specificita rekombinantní SmPOP byla dále zkoumána pomocí panelu inhibitorů s aldehydovou reakční skupinou s variabilní aminokyselinou v pozici P_2 (Z-Xaa-Pro-CHO). Inhibiční profil odpovídal substrátovému profilu s výjimkou pro inhibitor s Pro v P_2 pozici (Z-Pro-Pro-CHO), který byl účinným inhibitorem, ačkoliv peptidový substrát (Z-Pro-Pro-ACC) nebyl štěpen.

Antischistosomální účinek inhibitorů SmPOP byl následně testován *ex vivo* na živých schistosomulách. Tato analýza ukázala, že některé z inhibitorů způsobily silnou degeneraci až mortalitu. Z těchto výsledků vyplývá, že SmPOP je kritická pro přežití parazita a je potenciálním cílem pro vývoj antischistosomálních terapeutik na bázi malých inhibitorů.
RT-qPCR a substrátová analýza potvrdila, že SmPOP je exprimována ve stádiích krevničky, které žijí v lidském hostiteli (dospělci, schistosomuly a vejce). SmPOP byla imunolokalizována na tegumentu dospělců parazita. Naproti tomu nebyla nalezena

v gastrodermis a lumen střeva, což naznačuje, že SmPOP se nepodílí na trávení krevniček ale má spíše specifické role na rozhraní hostitele a parazita. Obdobná lokalizace v tegumentu byla nalezena i pro cysteinovou proteasu *S. mansoni* katepsin B2, jehož fyziologická funkce nebyla zatím objasněna (Caffrey et al. 2002).

Distribuce SmPOP byla studována pomocí aktivitního testu na živých krevničkách a na jejich ES produktech. Tato analýza ukázala, že SmPOP je aktivní enzym na povrchu parazita a není sekretován do hostitelského prostředí. Dále bylo prokázáno, že SmPOP štěpí *in vitro* vasoregulační peptidy angiotensin I a bradykinin. Tyto peptidy byly stejným způsobem štěpeny i v testech *ex vivo* s dospělými krevničkami. Angiotensin I je produkován renin-angiotensinovým systémem, který je primární fyziologický regulátor krevního tlaku v lidském těle (Campbell 2003). SmPOP štěpí angiotensin I na vasodilatační angiotensin-(1-7), který zároveň inhibuje proliferaci buněk, angiogenezi, fibrózu a zánět (Izumi and Iwao, 2006; Gallagher et al 2013). Bradykinin je generován systémem kalikrein-kinin, který se rovněž podílí na regulaci krevního tlaku (Campbell 2003). Bradykinin je silný vazodilatátor, který podporuje cevní permeabilitu a působí diureticky, a proteolytické působení SmPOP jej inaktivuje. Podle těchto výsledků lze předpokládat, že SmPOP lokalizovaná na tegumentu parazita přispívá k modulaci nebo dysregulaci homeostatických systémů řízených peptidovými hormony a tím přispívá k přežití parazita v krevním systému hostitele.

6. Závěry

Disertační práce se zabývala proteolytickými enzymy z krevničky střevní (*Schistosoma mansoni*), která způsobuje závažné parazitární onemocnění schistosomózu. Výsledky jsou shrnuty ve čtyřech publikacích a dvou připravovaných rukopisech, které jsou podávány do mezinárodního časopisu.

Disertační práce přináší nový ucelený popis sekretovaných proteas, které se významně účastní interakcí krevničky s lidským hostitelem. Dále jsou prezentovány nové poznatky o trávicích a povrchových proteasách krevničky, které byly v práci validovány jako vhodné molekulární cíle, a o jejich inhibitorech jako potenciálních antischistosomálních léčivech.

V disertační práci byly splněny zadané cíle a hlavní získané výsledky jsou následující:

1. Podle navrženého postupu byly připraveny exkrečně-sekreční (ES) produkty ze stádií krevničky parazitujících v lidském hostiteli (dospělců, schistosomul a vajec) v množství umožňujícím jejich analýzu.
2. S využitím přístupů funkční proteomiky byly popsány hlavní proteolytické aktivity ES produktů tří stádií krevničky. Pomocí dvou metod pro substrátovou analýzu (s fluorescenční detekcí a detekcí hmotnostní spektrometrií) byla identifikována sada unikátních aktivit endo- a exo-peptidas, které byly klasifikovány s využitím specifických inhibitorů selektivních pro jednotlivé třídy proteas. Data ukázala komplexní složení proteolytického systému ES produktů pro každé z vývojových stádií, s převažujícími serinovými a cysteinovými proteasami a metaloproteasami.
3. Pomocí bioinformatické analýzy byly v genomu krevničky identifikovány a anotovány geny pro 5 odlišných serinových proteas (SmSPs). RT-qPCR analýza jejich exprese v jednotlivých vývojových stadiích ukázala komplexní expresní profil pro jednotlivé SmSPs, přičemž nejvíce byla zastoupena proteasa SmSP2. Aktivity SmSPs byly také detekovány pomocí selektivních fluorogenních substrátů v homogenátech a ES produktech studovaných stádií.
4. Pomocí přístupu chemické genomiky byla identifikována sada proteas krevničky nezbytných pro životaschopnost parazita, které představují molekulární cíle pro vývoj nových inhibitorů s antischistosomální aktivitou. Konkrétně se jedná o trávicí proteasy SmCB, SmCC a SmCD a povrchovou proteasu SmPOP.

5. Proteasy SmPOP a SmCC byly připraveny rekombinantní expresí v bakteriích *E. coli* respektive prvoku *L. tarentolae* a purifikovány v aktivní formě. S těmito enzymy byla testována účinnost několika rozsáhlých sad syntetických inhibitorů pomocí kinetického testu *in vitro* s fluorogenními substráty.
6. Inhibitory účinné *in vitro* (bod 5) byly testovány *ex vivo* v biologickém testu na živých schistosomulách v kultivačním médiu. Tyto experimenty identifikovaly několik inhibitorů, které představují perspektivní templáty pro vývoj potenciálních léčiv proti schistosomóze.

7. Použitá literatura

Abdulla, M. H., K. C. Lim, M. Sajid, J. H. McKerrow, and C. R. Caffrey. 2007. 'Schistosomiasis mansoni: novel chemotherapy using a cysteine protease inhibitor', *PLoS Med*, 4: e14.

Abdulla, M. H., D. S. Ruelas, B. Wolff, J. Snedecor, K. C. Lim, F. Xu, A. R. Renslo, J. Williams, J. H. McKerrow, and C. R. Caffrey. 2009. 'Drug discovery for schistosomiasis: hit and lead compounds identified in a library of known drugs by medium-throughput phenotypic screening', *PLoS Negl Trop Dis*, 3: e478.

Alapati, K., D. Kesanakurti, J. S. Rao, and V. R. Dasari. 2014. 'uPAR and cathepsin B-mediated compartmentalization of JNK regulates the migration of glioma-initiating cells', *Stem Cell Res*, 12: 716-29.

Andrade, L. F., L. A. Nahum, L. G. Avelar, L. L. Silva, A. Zerlotini, J. C. Ruiz, and G. Oliveira. 2011. 'Eukaryotic protein kinases (ePKs) of the helminth parasite Schistosoma mansoni', *BMC Genomics*, 12: 215.

Bal, G., P. Van der Veken, D. Antonov, A. M. Lambeir, P. Grellier, S. L. Croft, K. Augustyns, and A. Haemers. 2003. 'Prolylisoxazoles: potent inhibitors of prolyloligopeptidase with antitrypanosomal activity', *Bioorg Med Chem Lett*, 13: 2875-8.

Banerjee, R., J. Liu, W. Beatty, L. Pelosof, M. Klemba, and D. E. Goldberg. 2002. 'Four plasmepsins are active in the Plasmodium falciparum food vacuole, including a protease with an active-site histidine', *Proceedings of the National Academy of Sciences of the United States of America*, 99: 990-95.

Barrett, A. J., A. A. Kembhavi, M. A. Brown, H. Kirschke, C. G. Knight, M. Tamai, and K. Hanada. 1982. 'L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L', *Biochem J*, 201: 189-98.

Barrett, A. J., and H. Kirschke. 1981. 'Cathepsin B, Cathepsin H, and cathepsin L', *Methods Enzymol*, 80 Pt C: 535-61.

Bastos, I. M., F. N. Motta, S. Charneau, J. M. Santana, L. Dubost, K. Augustyns, and P. Grellier. 2010. 'Prolyl oligopeptidase of Trypanosoma brucei hydrolyzes native collagen, peptide hormones and is active in the plasma of infected mice', *Microbes Infect*, 12: 457-66.

Benes, P., V. Vetvicka, and M. Fusek. 2008. 'Cathepsin D--many functions of one aspartic protease', *Crit Rev Oncol Hematol*, 68: 12-28.

Berriman, M., B. J. Haas, P. T. LoVerde, R. A. Wilson, G. P. Dillon, G. C. Cerqueira, S. T. Mashiyama, B. Al-Lazikani, L. F. Andrade, P. D. Ashton, M. A. Aslett, D. C. Bartholomeu, G. Blandin, C. R. Caffrey, A. Coghlan, R. Coulson, T. A. Day, A. Delcher, R. DeMarco, A. Djikeng, T. Eyre, J. A. Gamble, E. Ghedin, Y. Gu, C. Hertz-Fowler, H. Hirai, Y. Hirai, R. Houston, A. Ivens, D. A. Johnston, D. Lacerda, C. D. Macedo, P. McVeigh, Z. Ning, G. Oliveira, J. P. Overington, J. Parkhill, M. Pertea, R. J. Pierce, A. V. Protasio, M. A. Quail, M. A. Rajandream, J. Rogers, M. Sajid, S. L. Salzberg, M. Stanke, A. R. Tivey, O. White, D. L. Williams, J. Wortman, W. Wu, M. Zamanian, A. Zerlotini, C. M. Fraser-Liggett, B. G. Barrell, and N. M. El-Sayed. 2009. 'The genome of the blood fluke Schistosoma mansoni', *Nature*, 460: 352-8.

Bibo-Verdugo, B., A. J. O'Donoghue, L. Rojo-Arreola, C. S. Craik, and F. Garcia-Carreno. 2016. 'Complementary Proteomic and Biochemical Analysis of Peptidases in Lobster Gastric Juice Uncovers the Functional Role of Individual Enzymes in Food Digestion', *Mar Biotechnol (NY)*, 18: 201-14.

Blumberg, S., V. I. Teichberg, J. L. Charli, L. B. Hersh, and J. F. Mckelvy. 1980. 'Cleavage of Substance-P to an N-Terminal Tetrapeptide and a C-Terminal Heptapeptide by a Post-Proline Cleaving Enzyme from Bovine Brain', *Brain Research*, 192: 477-86.

Bogish, B., C. Carter, and T Oeltmann. 2012. *Human Parasitology* (Academic Press).

Bogitsh, B. J., J. P. Dalton, C. P. Brady, and P. J. Brindley. 2001. 'Gut-associated immunolocalization of the Schistosoma mansoni cysteine proteases, SmCL1 and SmCL2', *J Parasitol*, 87: 237-41.

Bogitsh, B. J., and M. H. Dresden. 1983. 'Fluorescent histochemistry of acid proteases in adult Schistosoma mansoni and Schistosoma japonicum', *J Parasitol*, 69: 106-10.

Boros, D. L. 1989. 'Immunopathology of Schistosoma mansoni infection', *Clin Microbiol Rev*, 2: 250-69.

Brady, C. P., A. J. Dowd, P. J. Brindley, T. Ryan, S. R. Day, and J. P. Dalton. 1999. 'Recombinant expression and localization of Schistosoma mansoni cathepsin L1 support its role in the degradation of host hemoglobin', *Infect Immun*, 67: 368-74.

Braschi, S., W. C. Borges, and R. A. Wilson. 2006. 'Proteomic analysis of the schistosome tegument and its surface membranes', *Mem Inst Oswaldo Cruz*, 101 Suppl 1: 205-12.

Breitling, R., S. Klingner, N. Callewaert, R. Pietrucha, A. Geyer, G. Ehrlich, R. Hartung, A. Muller, R. Contreras, S. M. Beverley, and K. Alexandrov. 2002. 'Non-pathogenic trypanosomatid protozoa as a platform for protein research and production', *Protein Expr Purif*, 25: 209-18.

Brindley, P. J., B. H. Kalinna, J. Y. Wong, B. J. Bogitsh, L. T. King, D. J. Smyth, C. K. Verity, G. Abbenante, R. I. Brinkworth, D. P. Fairlie, M. L. Smythe, P. J. Milburn, H. Bielefeldt-Ohmann, Y. Zheng, and D. P. McManus. 2001. 'Proteolysis of human hemoglobin by schistosome cathepsin D', *Mol Biochem Parasitol*, 112: 103-12.

Caffrey, C. R. 2007. 'Chemotherapy of schistosomiasis: present and future', *Curr Opin Chem Biol*, 11: 433-9.

Caffrey, C. R. 2015. 'Schistosomiasis and its treatment', Future Med Chem, 7: 675-6.

Caffrey, C. R., M. A. Mathieu, A. M. Gaffney, J. P. Salter, M. Sajid, K. D. Lucas, C. Franklin, M. Bogyo, and J. H. McKerrow. 2000. 'Identification of a cDNA encoding an active asparaginyl endopeptidase of Schistosoma mansoni and its expression in Pichia pastoris', *FEBS Lett*, 466: 244-8.

Caffrey, C. R., J. H. McKerrow, J. P. Salter, and M. Sajid. 2004. 'Blood 'n' guts: an update on schistosome digestive peptidases', *Trends Parasitol*, 20: 241-8.

Caffrey, C. R., L. Placha, C. Barinka, M. Hradilek, J. Dostal, M. Sajid, J. H. McKerrow, P. Majer, J. Konvalinka, and J. Vondrasek. 2005. 'Homology modeling and SAR analysis of Schistosoma japonicum cathepsin D (SjCD) with statin inhibitors identify a unique active site steric barrier with potential for the design of specific inhibitors', *Biol Chem*, 386: 339-49.

Caffrey, C. R., J. P. Salter, K. D. Lucas, D. Khiem, I. Hsieh, K. C. Lim, A. Ruppel, J. H. McKerrow, and M. Sajid. 2002. 'SmCB2, a novel tegumental cathepsin B from adult Schistosoma mansoni', *Mol Biochem Parasitol*, 121: 49-61.

Campbell, D. J. 2003. 'The renin-angiotensin and the kallikrein-kinin systems', *Int J Biochem Cell Biol*, 35: 784-91.

Carvalho, W. S., C. T. Lopes, L. Juliano, P. M. Coelho, J. R. Cunha-Melo, W. T. Beraldo, and J. L. Pesquero. 1998. 'Purification and partial characterization of kininogenase activity from Schistosoma mansoni adult worms', *Parasitology*, 117 (Pt 4): 311-9.

Cezari, M. H., L. Puzer, M. A. Juliano, A. K. Carmona, and L. Juliano. 2002. 'Cathepsin B carboxydipeptidase specificity analysis using internally quenched fluorescent peptides', *Biochem J*, 368: 365-9.

Cigic, B., S. W. Dahl, R. H. Pain. 2000. The residual pro-part of cathepsin C fulfills the criteria required for an intramolecular chaperone in folding and stabilizing the human proenzyme. Biochemistry, 39, 12382-12390.

Cioli, D., S. S. Botros, K. Wheatcroft-Francklow, A. Mbaye, V. Southgate, L. A. Tchuente, L. Pica-Mattoccia, A. R. Troiani, S. H. El-Din, A. N. Sabra, J. Albin, D. Engels, and M. J. Doenhoff. 2004. 'Determination of ED50 values for praziquantel in praziquantel-resistant and -susceptible Schistosoma mansoni isolates', *Int J Parasitol*, 34: 979-87.

Cioli, D., L. Pica-Mattoccia, and S. Archer. 1993. 'Drug resistance in schistosomes', *Parasitol Today*, 9: 162-6.

ClinicalTrials Home page: https://clinicaltrials.gov/ct2/show/NCT00870649?term=Bilhvax&rank=2 (2.6.2018)

Cocude, C., C. Pierrot, C. Cetre, J. Fontaine, C. Godin, A. Capron, and J. Khalife. 1999. 'Identification of a developmentally regulated Schistosoma mansoni serine protease homologous to mouse plasma kallikrein and human factor I', *Parasitology*, 118 (Pt 4): 389-96.

Correnti, J. M., P. J. Brindley, and E. J. Pearce. 2005. 'Long-term suppression of cathepsin B levels by RNA interference retards schistosome growth', *Mol Biochem Parasitol*, 143: 209-15.

Cunningham, D. F., and B. O'Connor. 1997. Proline specific peptidases. Biochim Biophys Acta 1343: 160–186

Dahl, S. W., T. Halkier, C. Lauritzen, I. Dolenc, J. Pedersen, V. Turk, and B. Turk. 2001. 'Human recombinant pro-dipeptidyl peptidase I (cathepsin C) can be activated by cathepsins L and S but not by autocatalytic processing', *Biochemistry*, 40: 1671-8.

Dalton, J. P., P. Skelly, and D. W. Halton. 2004. 'Role of the tegument and gut in nutrient uptake by parasitic platyhelminths', *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 82: 211-32.

Delcroix, M., M. Sajid, C. R. Caffrey, K. C. Lim, J. Dvorak, I. Hsieh, M. Bahgat, C. Dissous, and J. H. McKerrow. 2006. 'A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite', *J Biol Chem*, 281: 39316-29.

Deu, E., M. J. Leyva, V. E. Albrow, M. J. Rice, J. A. Ellman, and M. Bogyo. 2010. 'Functional studies of Plasmodium falciparum dipeptidyl aminopeptidase I using small molecule inhibitors and active site probes', *Chem Biol*, 17: 808-19.

Dissous, C., N. Khayath, J. Vicogne, and M. Capron. 2006. 'Growth factor receptors in helminth parasites: signalling and host-parasite relationships', *FEBS Lett*, 580: 2968-75.

Doenhoff, M. J., R. G. Stanley, D. Pryce, R. C. Curtis, H. Parry, K. Griffiths, and C. L. Jackson. 2003. 'Identification of a fibrinolytic enzyme in Schistosoma mansoni eggs and modulated blood fibrinogen metabolism in S. mansoni-infected mice', *Parasitology*, 126: 231-4.

Dolenc, I., B. Turk, J. Kos, and V. Turk. 1996. 'Interaction of human cathepsin C with chicken cystatin', *FEBS Lett*, 392: 277-80.

Dolenc, I., B. Turk, G. Pungercic, A. Ritonja, and V. Turk. 1995. 'Oligomeric structure and substrate induced inhibition of human cathepsin C', *J Biol Chem*, 270: 21626-31.

Dresdner, K., L. A. Barker, M. Orlowski, and S. Wilk. 1982. 'Subcellular distribution of prolyl endopeptidase and cation-sensitive neutral endopeptidase in rabbit brain', *J Neurochem*, 38: 1151-4.

Dvorak, J., and M. Horn. 2018. 'Serine proteases in schistosomes and other trematodes', *International Journal for Parasitology*, 48: 333-44.

Dvorak, J., S. T. Mashiyama, M. Sajid, S. Braschi, M. Delcroix, E. L. Schneider, W. H. McKerrow, M. Bahgat, E. Hansell, P. C. Babbitt, C. S. Craik, J. H. McKerrow, and C. R. Caffrey. 2009. 'SmCL3, a gastrodermal cysteine protease of the human blood fluke Schistosoma mansoni', *PLoS Negl Trop Dis*, 3: e449.

Fais, S. 2007. 'Cannibalism: a way to feed on metastatic tumors', *Cancer Lett*, 258: 155-64.

Fajtová, P. 2011. *Prolyl oligopeptidasa z krevničky Schistosoma mansoni*. Praha. Diplomová práce. Univerzita Karlova v Praze. Přírodovědecká fakulta.

Fallon, P. G., and M. J. Doenhoff. 1994. 'Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in Schistosoma mansoni in mice is drug specific', *Am J Trop Med Hyg*, 51: 83-8.

Förstl, M., L. Kolářová, M. Konštacký, Z. Veselský, P. Macek, and P. Dvořák. 2003. 'RIZIKO IMPORTU SCHISTOSOMÓZY (BILHARZIÓZY)'. https://www.internimedicina.cz/pdfs/int/2003/02/03.pdf (6.5.2018).

Fulop, V., Z. Bocskei, and L. Polgar. 1998. 'Prolyl oligopeptidase: an unusual beta-propeller domain regulates proteolysis', *Cell*, 94: 161-70.

Gallagher P., E., A., L., Arter, B., Krishnan, M., A., Garcia-Espinosa, A. Tallant. 2013. Angiotensin II/Angiotensin-(1–7). In: Katin AJ, editor. Handbook of Biologically Active Peptides. pp. 494–

501.Garcia-Horsman, J. A., P. T. Mannisto, and J. I. Venalainen. 2007. 'On the role of prolyl oligopeptidase in health and disease', *Neuropeptides*, 41: 1-24.

Githui, E. K., R. T. Damian, R. A. Aman, M. A. Ali, and J. M. Kamau. 2009. 'Schistosoma spp.: Isolation of microtubule associated proteins in the tegument and the definition of dynein light chains components', *Exp Parasitol*, 121: 96-104.

Gobert, G. N., D. J. Stenzel, D. P. McManus, and M. K. Jones. 2003. 'The ultrastructural architecture of the adult Schistosoma japonicum tegument', *Int J Parasitol*, 33: 1561-75.

Gotoh, H., M. Hagihara, T. Nagatsu, H. Iwata, and T. Miura. 1988. 'Activity of dipeptidyl peptidase IV and post-proline cleaving enzyme in sera from osteoporotic patients', *Clin Chem*, 34: 2499-501.

Gryseels, B. 2000. 'Schistosomiasis vaccines: the Devils' Advocate's final plea', *Parasitol Today*, 16: 357-8.

Gryseels, B., K. Polman, J. Clerinx, and L. Kestens. 2006. 'Human schistosomiasis', *Lancet*, 368: 1106-18.

Haas, W., S. Haeberlein, S. Behring, and E. Zoppelli. 2008. 'Schistosoma mansoni: human skin ceramides are a chemical cue for host recognition of cercariae', *Exp Parasitol*, 120: 94-7.

Hall, S. L., S. Braschi, M. Truscott, W. Mathieson, I. M. Cesari, and R. A. Wilson. 2011. 'Insights into blood feeding by schistosomes from a proteomic analysis of worm vomitus', *Mol Biochem Parasitol*, 179: 18-29.

Harnett, W., and J. R. Kusel. 1986. 'Increased exposure of parasite antigens at the surface of adult male Schistosoma mansoni exposed to praziquantel in vitro', *Parasitology*, 93 (Pt 2): 401-5.

Harris, J. L., B. J. Backes, F. Leonetti, S. Mahrus, J. A. Ellman, and C. S. Craik. 2000. 'Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries', *Proc Natl Acad Sci U S A*, 97: 7754-9.

Hasnain, S., T. Hirama, C. P. Huber, P. Mason, and J. S. Mort. 1993. 'Characterization of cathepsin B specificity by site-directed mutagenesis. Importance of Glu245 in the S2-P2 specificity for arginine and its role in transition state stabilization', *J Biol Chem*, 268: 235-40.

Hola-Jamriska, L., J. P. Dalton, J. Aaskov, and P. J. Brindley. 1999. 'Dipeptidyl peptidase I and III activities of adult schistosomes', *Parasitology*, 118 (Pt 3): 275-82.

Hola-Jamriska, L., L. T. King, J. P. Dalton, V. H. Mann, J. G. Aaskov, and P. J. Brindley. 2000. 'Functional expression of dipeptidyl peptidase I (Cathepsin C) of the oriental blood fluke Schistosoma japonicum in Trichoplusia ni insect cells', *Protein Expr Purif*, 19: 384-92.

Hola-Jamriska, L., J. F. Tort, J. P. Dalton, S. R. Day, J. Fan, J. Aaskov, and P. J. Brindley. 1998. 'Cathepsin C from Schistosoma japonicum--cDNA encoding the preproenzyme and its phylogenetic relationships', *Eur J Biochem*, 255: 527-34.

Horn, M., M. Baudys, Z. Voburka, I. Kluh, J. Vondrasek, and M. Mares. 2002. 'Free-thiol Cys331 exposed during activation process is critical for native tetramer structure of cathepsin C (dipeptidyl peptidase I)', *Protein Sci*, 11: 933-43.

Horn, M., A. Jilkova, M. Mares. 2014. Katepsinové proteasy v patologii. Chemické listy, 4, 358-363

Horn, M., M. Nussbaumerova, M. Sanda, Z. Kovarova, J. Srba, Z. Franta, D. Sojka, M. Bogyo, C. R. Caffrey, P. Kopacek, and M. Mares. 2009. 'Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by functional proteomics', *Chem Biol*, 16: 1053-63.

Hotez, P. J., and A. Fenwick. 2009. 'Schistosomiasis in Africa: an emerging tragedy in our new global health decade', *PLoS Negl Trop Dis*, 3: e485.

Hotez, P. J., and A. Kamath. 2009. 'Neglected tropical diseases in sub-saharan Africa: review of their prevalence, distribution, and disease burden', *PLoS Negl Trop Dis*, 3: e412.

Hotez, P. J., D. H. Molyneux, A. Fenwick, J. Kumaresan, S. E. Sachs, J. D. Sachs, and L. Savioli. 2007. 'Control of neglected tropical diseases', *N Engl J Med*, 357: 1018-27.

Huber, R., and W. Bode. 1979 Structural Basis of the Activation, Action and Inhibition of Trypsin. Hoppe-Seylers Zeitschrift Fur Physilogische Chemie 360: 489–489.

Chappell, C. L., and M. H. Dresden. 1986. 'Schistosoma mansoni: proteinase activity of "hemoglobinase" from the digestive tract of adult worms', *Exp Parasitol*, 61: 160-7.

Chen, J. M., P. M. Dando, N. D. Rawlings, M. A. Brown, N. E. Young, R. A. Stevens, E. Hewitt, C. Watts, and A. J. Barrett. 1997. 'Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase', *J Biol Chem*, 272: 8090-8.

Chitsulo, L., D. Engels, A. Montresor, and L. Savioli. 2000. 'The global status of schistosomiasis and its control', *Acta Trop*, 77: 41-51.

Ingram, J. R., S. B. Rafi, A. A. Eroy-Reveles, M. Ray, L. Lambeth, I. Hsieh, D. Ruelas, K. C. Lim, J. Sakanari, C. S. Craik, M. P. Jacobson, and J. H. McKerrow. 2012. 'Investigation of the proteolytic functions of an expanded cercarial elastase gene family in Schistosoma mansoni', *PLoS Negl Trop Dis*, 6: e1589.

Ismail, M., S. Botros, A. Metwally, S. William, A. Farghally, L. F. Tao, T. A. Day, and J. L. Bennett. 1999. 'Resistance to praziquantel: direct evidence from Schistosoma mansoni isolated from Egyptian villagers', *Am J Trop Med Hyg*, 60: 932-5.

Izumi, Y. and H. Iwao. 2006. Angiotensin II and Its Related Peptides. In: Katin AJ, editor. Handbook of Biologically Active Peptides, pp. 1169–1174.

Jamieson, B., G., M. 2016, Schistosoma. Biology, Pathology and Control, první vydání. Boca Raton : CRC Press/Taylor & Francis, 515 stran. ISBN: 9781498744256

Jena Bioscience manuál: https://www.jenabioscience.com/images/ae3a4f50f1/EGE-1310.pdf (1.9.2016)

Jeziorski, M. C., and R. M. Greenberg. 2006. 'Voltage-gated calcium channel subunits from platyhelminths: potential role in praziquantel action', *Int J Parasitol*, 36: 625-32.

Jilkova, A., M. Horn, P. Rezacova, L. Maresova, P. Fajtova, J. Brynda, J. Vondrasek, J. H. McKerrow, C. R. Caffrey, and M. Mares. 2014. 'Activation route of the Schistosoma mansoni cathepsin B1 drug target: structural map with a glycosaminoglycan switch', *Structure*, 22: 1786-98.

Jilkova, A., P. Rezacova, M. Lepsik, M. Horn, J. Vachova, J. Fanfrlik, J. Brynda, J. H. McKerrow, C. R. Caffrey, and M. Mares. 2011. 'Structural basis for inhibition of cathepsin B drug target from the human blood fluke, Schistosoma mansoni', *J Biol Chem*, 286: 35770-81.

Jolly, E. R., C. S. Chin, S. Miller, M. M. Bahgat, K. C. Lim, J. DeRisi, and J. H. McKerrow. 2007. 'Gene expression patterns during adaptation of a helminth parasite to different environmental niches', *Genome Biol*, 8: R65.

Jones, M. K., G. N. Gobert, L. Zhang, P. Sunderland, and D. P. McManus. 2004. 'The cytoskeleton and motor proteins of human schistosomes and their roles in surface maintenance and host-parasite interactions', *Bioessays*, 26: 752-65.

Kasny, M., L. Mikes, V. Hampl, J. Dvorak, C. R. Caffrey, J. P. Dalton, and P. Horak. 2009. 'Chapter 4. Peptidases of trematodes', *Adv Parasitol*, 69: 205-97.

Kato, T., M. Okada, and T. Nagatsu. 1980. 'Distribution of post-proline cleaving enzyme in human brain and the peripheral tissues', *Mol Cell Biochem*, 32: 117-21.

Kirschke, H., A. J. Barrett, and N. D. Rawlings. 1995. 'Proteinases 1: lysosomal cysteine proteinases', *Protein Profile*, 2: 1581-643.

Kiss, A. L., Z. Szeltner, V. Fulop, and L. Polgar. 2004. 'His507 of acylaminoacyl peptidase stabilizes the active site conformation, not the catalytic intermediate', *FEBS Lett*, 571: 17-20.

Klinkert, M. Q., K. Bommert, D. Moser, R. Felleisen, G. Link, O. Doumbo, and E. Beck. 1991. 'Immunological analysis of cloned Schistosoma mansoni antigens Sm31 and Sm32 with sera of schistosomiasis patients', *Trop Med Parasitol*, 42: 319-24.

Kloetzel, K., and R. M. Lewert. 1966. 'Pigment formation in Schistosoma mansoni infections in the white mouse', *Am J Trop Med Hyg*, 15: 28-31.

Kominami, E., K. Ishido, D. Muno, and N. Sato. 1992. 'The primary structure and tissue distribution of cathepsin C', *Biol Chem Hoppe Seyler*, 373: 367-73.

Lawandi, J., S. Gerber-Lemaire, L. Juillerat-Jeanneret, and N. Moitessier. 2010. 'Inhibitors of prolyl oligopeptidases for the therapy of human diseases: defining diseases and inhibitors', *J Med Chem*, 53: 3423-38.

Li, H., E. L. Ponder, M. Verdoes, K. H. Asbjornsdottir, E. Deu, L. E. Edgington, J. T. Lee, C. J. Kirk, S. D. Demo, K. C. Williamson, and M. Bogyo. 2012. 'Validation of the proteasome as a therapeutic

target in Plasmodium using an epoxyketone inhibitor with parasite-specific toxicity', *Chem Biol*, 19: 1535-45.

Losada, S., N. Chacon, C. Colmenares, H. Bermudez, A. Lorenzo, J. P. Pointier, A. Theron, B. Alarcon de Noya, and O. Noya. 2005. 'Schistosoma: cross-reactivity and antigenic community among different species', *Exp Parasitol*, 111: 182-90.

Loukas, A., M. Tran, and M. S. Pearson. 2007. 'Schistosome membrane proteins as vaccines', *Int J Parasitol*, 37: 257-63.

Maes, M., F. Goossens, S. Scharpe, J. Calabrese, R. Desnyder, and H. Y. Meltzer. 1995. 'Alterations in Plasma Prolyl Endopeptidase Activity in Depression, Mania, and Schizophrenia - Effects of Antidepressants, Mood Stabilizers, and Antipsychotic-Drugs', *Psychiatry Research*, 58: 217-25.

Mair, G. R., A. G. Maule, B. Fried, T. A. Day, and D. W. Halton. 2003. 'Organization of the musculature of schistosome cercariae', *J Parasitol*, 89: 623-5.

Mallen-St Clair, J., C. T. Pham, S. A. Villalta, G. H. Caughey, and P. J. Wolters. 2004. 'Mast cell dipeptidyl peptidase I mediates survival from sepsis', *J Clin Invest*, 113: 628-34.

McCarthy, E., C. Stack, S. M. Donnelly, S. Doyle, V. H. Mann, P. J. Brindley, M. Stewart, T. A. Day, A. G. Maule, and J. P. Dalton. 2004. 'Leucine aminopeptidase of the human blood flukes, Schistosoma mansoni and Schistosoma japonicum', *Int J Parasitol*, 34: 703-14.

McGuire, M. J., P. E. Lipsky, and D. L. Thiele. 1992. 'Purification and characterization of dipeptidyl peptidase I from human spleen', *Arch Biochem Biophys*, 295: 280-8.

McKerrow, J. H. 2005. 'Designing drugs for parasitic diseases of the developing world', *PLoS Med*, 2: e210.

McKerrow, J. H., C. Caffrey, B. Kelly, P. Loke, and M. Sajid. 2006. 'Proteases in parasitic diseases', *Annu Rev Pathol*, 1: 497-536.

McKerrow, J. H., P. S. Doyle, J. C. Engel, L. M. Podust, S. A. Robertson, R. Ferreira, T. Saxton, M. Arkin, I. D. Kerr, L. S. Brinen, and C. S. Craik. 2009. 'Two approaches to discovering and developing new drugs for Chagas disease', *Mem Inst Oswaldo Cruz*, 104 Suppl 1: 263-9.

McLaren, D. J., and D. J. Hockley. 1977. 'Blood flukes have a double outer membrane', *Nature*, 269: 147-9.

McManus, D. P., and A. Loukas. 2008. 'Current status of vaccines for schistosomiasis', *Clin Microbiol Rev*, 21: 225-42.

Melman, S. D., M. L. Steinauer, C. Cunningham, L. S. Kubatko, I. N. Mwangi, N. B. Wynn, M. W. Mutuku, D. M. Karanja, D. G. Colley, C. L. Black, W. E. Secor, G. M. Mkoji, and E. S. Loker. 2009. 'Reduced susceptibility to praziquantel among naturally occurring Kenyan isolates of Schistosoma mansoni', *PLoS Negl Trop Dis*, 3: e504.

MEROPS Home page: https://www.ebi.ac.uk/merops (1.8.2018)

Michel, A., H. Ghoneim, M. Resto, M. Q. Klinkert, and W. Kunz. 1995. 'Sequence, characterization and localization of a cysteine proteinase cathepsin L in Schistosoma mansoni', *Mol Biochem Parasitol*, 73: 7-18.

Mort, J. S., and D. J. Buttle. 1997. 'Cathepsin B', *Int J Biochem Cell Biol*, 29: 715-20.

Muller, R. 2002. *Worms and Human Disease* (CABI Publishing).

Murata, M., S. Miyashita, C. Yokoo, M. Tamai, K. Hanada, K. Hatayama, T. Towatari, T. Nikawa, and N. Katunuma. 1991. 'Novel epoxysuccinyl peptides. Selective inhibitors of cathepsin B, in vitro', *FEBS Lett*, 280: 307-10.

Musil, D., D. Zucic, D. Turk, R. A. Engh, I. Mayr, R. Huber, T. Popovic, V. Turk, T. Towatari, N. Katunuma, and et al. 1991. 'The refined 2.15 A X-ray crystal structure of human liver cathepsin B: the structural basis for its specificity', *EMBO J*, 10: 2321-30.

Nakajima, T., Y. Ono, A. Kato, J. Maeda, and T. Ohe. 1992. 'Y-29794--a non-peptide prolyl endopeptidase inhibitor that can penetrate into the brain', *Neurosci Lett*, 141: 156-60.

Neitzel, J. J. 2010 Enzyme Catalysis: The Serine Proteases . Nature Education 3(9):21

Němečková, V., and L. Kolářová. 2004. 'Schistosoma - motolice v lékařské praxi'. https://www.internimedicina.cz/pdfs/int/2004/05/02.pdf.

Novinec, M., and B. Lenarcic. 2013. 'Papain-like peptidases: structure, function, and evolution', *Biomol Concepts*, 4: 287-308.

O'Donoghue, A. J., A. A. Eroy-Reveles, G. M. Knudsen, J. Ingram, M. Zhou, J. B. Statnekov, A. L. Greninger, D. R. Hostetter, G. Qu, D. A. Maltby, M. O. Anderson, J. L. Derisi, J. H. McKerrow, A. L. Burlingame, and C. S. Craik. 2012. 'Global identification of peptidase specificity by multiplex substrate profiling', *Nat Methods*, 9: 1095-100.

Page, M. J., and E. Di Cera. 2008. 'Serine peptidases: classification, structure and function', *Cell Mol Life Sci*, 65: 1220-36.

Planchart, S., R. N. Incani, and I. M. Cesari. 2007. 'Preliminary characterization of an adult worm "vomit" preparation of Schistosoma mansoni and its potential use as antigen for diagnosis', *Parasitol Res*, 101: 301-9.

Polgar, L. 1992. 'Structural relationship between lipases and peptidases of the prolyl oligopeptidase family', *FEBS Lett*, 311: 281-4.

Polgar, L. 2002. 'The prolyl oligopeptidase family', *Cell Mol Life Sci*, 59: 349-62.

Protasio, A. V., I. J. Tsai, A. Babbage, S. Nichol, M. Hunt, M. A. Aslett, N. De Silva, G. S. Velarde, T. J. Anderson, R. C. Clark, C. Davidson, G. P. Dillon, N. E. Holroyd, P. T. LoVerde, C. Lloyd, J. McQuillan, G. Oliveira, T. D. Otto, S. J. Parker-Manuel, M. A. Quail, R. A. Wilson, A. Zerlotini, D. W. Dunne, and M. Berriman. 2012. 'A systematically improved high quality genome and transcriptome of the human blood fluke Schistosoma mansoni', *PLoS Negl Trop Dis*, 6: e1455.

Rawlings, N. D., A. J. Barrett, and R. Finn. 2016. 'Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors', *Nucleic Acids Res*, 44: D343-50.

Rawlings, N. D., A. J. Barrett, and A., Bateman. 2012. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 40: D343–D350.

Riveau, G., D. Deplanque, F. Remoue, A. M. Schacht, H. Vodougnon, M. Capron, M. Thiry, J. Martial, C. Libersa, and A. Capron. 2012. 'Safety and immunogenicity of rSh28GST antigen in humans: phase 1 randomized clinical study of a vaccine candidate against urinary schistosomiasis', *PLoS Negl Trop Dis*, 6: e1704.

Roberts, L. S., and J. Janovy. 2006. *Foundation of Parasitology*.

Ruppel, A., H. J. Diesfeld, and U. Rother. 1985. 'Immunoblot analysis of Schistosoma mansoni antigens with sera of schistosomiasis patients: diagnostic potential of an adult schistosome polypeptide', *Clin Exp Immunol*, 62: 499-506.

Ruppel, A., Y. E. Shi, D. X. Wei, and H. J. Diesfeld. 1987. 'Sera of Schistosoma japonicum-infected patients cross-react with diagnostic 31/32 kD proteins of S. mansoni', *Clin Exp Immunol*, 69: 291-8.

Sabah, A. A., C. Fletcher, G. Webbe, and M. J. Doenhoff. 1986. 'Schistosoma mansoni: chemotherapy of infections of different ages', *Exp Parasitol*, 61: 294-303.

Sajid, M., J. H. McKerrow, E. Hansell, M. A. Mathieu, K. D. Lucas, I. Hsieh, D. Greenbaum, M. Bogyo, J. P. Salter, K. C. Lim, C. Franklin, J. H. Kim, and C. R. Caffrey. 2003. 'Functional expression and characterization of Schistosoma mansoni cathepsin B and its trans-activation by an endogenous asparaginyl endopeptidase', *Mol Biochem Parasitol*, 131: 65-75.

Salter, J. P., K. C. Lim, E. Hansell, I. Hsieh, and J. H. McKerrow. 2000. 'Schistosome invasion of human skin and degradation of dermal elastin are mediated by a single serine protease', *J Biol Chem*, 275: 38667-73.

Sharma, K. K., and B. J. Ortwerth. 1994. 'Purification and characterization of prolyl oligopeptidase from bovine lens', *Exp Eye Res*, 59: 107-15.

Sharma, V., P. Panwar, A. J. O'Donoghue, H. Cui, R. V. Guido, C. S. Craik, and D. Bromme. 2015. 'Structural requirements for the collagenase and elastase activity of cathepsin K and its selective inhibition by an exosite inhibitor', *Biochem J*, 465: 163-73.

Schadewaldt, H. 1962. '[Theodor BILHARZ (1825-1862), one of the founders of German tropical medicine research]', *Munch Med Wochenschr*, 104: 1730-4.

Schechter, I. 2005. 'Mapping of the active site of proteases in the 1960s and rational design of inhibitors/drugs in the 1990s', *Curr Protein Pept Sci*, 6: 501-12.

Skelly, P. J., and R. Alan Wilson. 2006. 'Making sense of the schistosome surface', *Adv Parasitol*, 63: 185-284.

Skelly, P. J., A. A. Da'dara, X. H. Li, W. Castro-Borges, and R. A. Wilson. 2014. 'Schistosome feeding and regurgitation', *PLoS Pathog*, 10: e1004246.

Skelly, P. J., J. W. Kim, J. Cunningham, and C. B. Shoemaker. 1994. 'Cloning, characterization, and functional expression of cDNAs encoding glucose transporter proteins from the human parasite Schistosoma mansoni', *J Biol Chem*, 269: 4247-53.

Small, J. L., A. J. O'Donoghue, E. C. Boritsch, O. V. Tsodikov, G. M. Knudsen, O. Vandal, C. S. Craik, and S. Ehrt. 2013. 'Substrate specificity of MarP, a periplasmic protease required for resistance to acid and oxidative stress in Mycobacterium tuberculosis', *J Biol Chem*, 288: 12489-99.

Sojka, D., D. Hartmann, P. Bartosova-Sojkova, and J. Dvorak. 2016. 'Parasite Cathepsin D-Like Peptidases and Their Relevance as Therapeutic Targets', Trends Parasitol, 32: 708-23.

Státní ústav pro kontrolu léčiv: http://www.sukl.cz/modules/medication/search.php?data[atc_group]=P (3.6.2018).

Steinmann, P., J. Keiser, R. Bos, M. Tanner, and J. Utzinger. 2006. 'Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk', *Lancet Infect Dis*, 6: 411-25.

Stirewalt, M. A., and C. H. Dorsey. 1974. 'Schistosoma manonsi: cercarial penetration of host epidermis at the ultrastructural level', *Exp Parasitol*, 35: 1-15.

Szeltner, Z., D. Rea, V. Renner, V. Fulop, and L. Polgar. 2002. 'Electrostatic effects and binding determinants in the catalysis of prolyl oligopeptidase. Site specific mutagenesis at the oxyanion binding site', *J Biol Chem*, 277: 42613-22.

Taylor, A. B., L. Pica-Mattoccia, C. M. Polcaro, E. Donati, X. Cao, A. Basso, A. Guidi, A. R. Rugel, S. P. Holloway, T. J. Anderson, P. J. Hart, D. Cioli, and P. T. LoVerde. 2015. 'Structural and Functional Characterization of the Enantiomers of the Antischistosomal Drug Oxamniquine', PLoS Negl Trop Dis, 9: e0004132.

Thetiot-Laurent, S. A., J. Boissier, A. Robert, and B. Meunier. 2013. 'Schistosomiasis chemotherapy', *Angew Chem Int Ed Engl*, 52: 7936-56.

Toide, K., M. Shinoda, T. Fujiwara, and Y. Iwamoto. 1997. 'Effect of a novel prolyl endopeptidase inhibitor, JTP-4819, on spatial memory and central cholinergic neurons in aged rats', *Pharmacology Biochemistry and Behavior*, 56: 427-34.

Tsai, I. J., T. D. Otto, and M. Berriman. 2010. 'Improving draft assemblies by iterative mapping and assembly of short reads to eliminate gaps', *Genome Biol*, 11: R41.

Turk, B., D. Turk, I. Dolenc, D. Turk, V. Turk. 2004. Dipeptidyl-peptidase I. In: Handbook of Proteolytic Enzymes. 2nd ed., (Barrett A.J., Rawlings N.D., Woessner J.F., eds.), Elsevier, Amsterdam, The Netherlands, 1192–1196.

Turk, D., V. Janjic, I. Stern, M. Podobnik, D. Lamba, S. W. Dahl, C. Lauritzen, J. Pedersen, V. Turk, and B. Turk. 2001. 'Structure of human dipeptidyl peptidase I (cathepsin C): exclusion domain added to an endopeptidase framework creates the machine for activation of granular serine proteases', *EMBO J*, 20: 6570-82.

Verjovski-Almeida, S., R. DeMarco, E. A. Martins, P. E. Guimaraes, E. P. Ojopi, A. C. Paquola, J. P. Piazza, M. Y. Nishiyama, Jr., J. P. Kitajima, R. E. Adamson, P. D. Ashton, M. F. Bonaldo, P. S. Coulson, G. P. Dillon, L. P. Farias, S. P. Gregorio, P. L. Ho, R. A. Leite, L. C. Malaquias, R. C. Marques, P. A. Miyasato, A. L. Nascimento, F. P. Ohlweiler, E. M. Reis, M. A. Ribeiro, R. G. Sa, G. C. Stukart, M. B. Soares, C. Gargioni, T. Kawano, V. Rodrigues, A. M. Madeira, R. A. Wilson, C. F. Menck, J. C. Setubal, L. C. Leite, and E. Dias-Neto. 2003. 'Transcriptome analysis of the acoelomate human parasite Schistosoma mansoni', *Nat Genet*, 35: 148-57.

Vigneswaran, N., W. Zhao, A. Dassanayake, S. Muller, D. M. Miller, and W. Zacharias. 2000. 'Variable expression of cathepsin B and D correlates with highly invasive and metastatic phenotype of oral cancer', *Hum Pathol*, 31: 931-7.

Volf, P., P. Horák, and et kol. 2007. *Paraziti a jejich biologie* (Triton).

Wang, Q., A. A. Da'dara, and P. J. Skelly. 2018. 'The blood fluke Schistosoma mansoni cleaves the coagulation protein high molecular weight kininogen (HK) but does not generate the vasodilator bradykinin', *Parasit Vectors*, 11: 182.

Welches, W. R., K. B. Brosnihan, and C. M. Ferrario. 1993. 'A Comparison of the Properties and Enzymatic-Activities of 3 Angiotensin Processing Enzymes - Angiotensin-Converting Enzyme, Prolyl Endopeptidase and Neutral Endopeptidase 24.11', *Life Sciences*, 52: 1461-80.

Wilk, S. 1983. 'Prolyl endopeptidase', *Life Sci*, 33: 2149-57.

Wilson, R. A. 2012. 'The cell biology of schistosomes: a window on the evolution of the early metazoa', *Protoplasma*, 249: 503-18.

World Health Organization: http://www.who.int/news-room/fact-sheets/detail/schistosomiasis (2.6.2018).

Yang, W. E., C. C. Ho, S. F. Yang, S. H. Lin, K. T. Yeh, C. W. Lin, and M. K. Chen. 2016. 'Cathepsin B Expression and the Correlation with Clinical Aspects of Oral Squamous Cell Carcinoma', *PLoS One*, 11: e0152165.

Yoshimoto, T., K. Kawahara, F. Matsubara, K. Kado, and D. Tsuru. 1985. 'Comparison of inhibitory effects of prolinal-containing peptide derivatives on prolyl endopeptidases from bovine brain and Flavobacterium', *J Biochem*, 98: 975-9.

Yoshimoto, T., W. H. Simmons, T. Kita, and D. Tsuru. 1981. 'Post-Proline Cleaving Enzyme from Lamb Brain', *Journal of Biochemistry*, 90: 325-34.

Young, N. D., A. R. Jex, B. Li, S. Liu, L. Yang, Z. Xiong, Y. Li, C. Cantacessi, R. S. Hall, X. Xu, F. Chen, X. Wu, A. Zerlotini, G. Oliveira, A. Hofmann, G. Zhang, X. Fang, Y. Kang, B. E. Campbell, A. Loukas, S. Ranganathan, D. Rollinson, G. Rinaldi, P. J. Brindley, H. Yang, J. Wang, J. Wang, and R. B. Gasser. 2012. 'Whole-genome sequence of Schistosoma haematobium', *Nat Genet*, 44: 221-5.

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Research paper

Excretion/secretion products from *Schistosoma mansoni* adults, eggs and schistosomula have unique peptidase specificity profiles

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ABSTRACT

Schistosomiasis is one of a number of chronic helminth diseases of poverty that severely impact personal and societal well-being and productivity. Peptidases (proteases) are vital to successful parasitism, and can modulate host physiology and immunology. Interference of peptidase action by specific drugs or vaccines can be therapeutically beneficial. To date, research on peptidases in the schistosome parasite has focused on either the functional characterization of individual peptidases or their annotation as part of global genome or transcriptome studies. We were interested in functionally characterizing the complexity of peptidase activity operating at the host–parasite interface, therefore the excretory-secretory products of key developmental stages of *Schistosoma mansoni* that parasitize the human were examined. Using class specific peptidase inhibitors in combination with a multiplex substrate profiling assay, a number of unique activities derived from endo- and exo-peptidases were revealed in the excretory-secretory products of schistosomula (larval migratory worms), adults and eggs. The data highlight the complexity of the functional degradome for each developmental stage of this parasite and facilitate further enquiry to establish peptidase identity, physiological and immunological function, and utility as drug or vaccine candidates.

1. Introduction

Schistosomiasis caused by the *Schistosoma* blood fluke is a chronic disease of poverty infecting more than 200 million people with as many as 800 million people at risk [1,2]. Schistosome larvae (cercariae), released into freshwater by intermediate snail hosts, penetrate human skin and subsequently develop into adult male or female worms in the host vascular system. Pairs of worms can survive for years, if not decades [3] and release many hundreds of eggs a day [4]. These eggs induce immune-inflammation and tissue damage that contribute to disease morbidity [5]. The disease hampers growth and development of children and severely impacts the ability of families to provide for themselves [6–8].

Proteolysis is a fundamental physiologic process contributing to both health and disease [9,10]. *Schistosoma* peptidases (a.k.a. proteases or proteolytic enzymes) are vital to successful parasitism, and facilitate invasion of the host, digestion of host proteins,

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reproduction, modulation of the host's physiology [11–19] and immune response [14,20]. Interference with these mechanisms by specific vaccines [21,22] or drugs may provide therapeutic benefits. Indeed, peptidases are excellent druggable targets [23–25] and a large body of literature exists demonstrating the therapeutic benefits of small molecule inhibitors targeting peptidases of schistosomes [26–29] and other infectious organisms [30–36].

Research on parasite peptidases has traditionally been driven by investigator interest in a particular molecule or class of molecules. For example, much of the considerable research focused on schistosome cysteine cathepsin enzymes as drug or vaccine targets has been facilitated by often inexpensive, sensitive and easy-to-use tools that include peptidyl substrates, inhibitors and antibodies. Detailed molecular, structural and immunological characterizations of these enzymes [29,37,38] have been greatly aided by their straightforward 'expressability' in heterologous systems such as yeast [38–41]. Over the last 10 years, the increasing availability of accurately annotated genomic [42–47] and transcriptomic data [48–53] has expanded our view of the number and complexity of peptidases (the 'degradome') expressed by the schistosome and how their expression is regulated throughout the parasite's life cycle. In parallel, a number of studies have generated (sub)proteome data for schistosomes (for reviews see Refs. [54,55]), including for the parasite's tegument (surface) [54,56–58] and excretory/secretory (ES) products [59,60]. Though fundamentally informative, these studies do not provide information on which peptidases are functionally active, including at the host–parasite interface.

We performed a global and unbiased analysis of peptidase activity and specificity in the ES products of key *Schistosoma mansoni* developmental stages residing in the human host, namely schistosomula (post-infective migratory larvae), adults and their eggs. We chose to examine ES products as these would contain peptidases more likely to operate at the host–parasite interface rather than extracts which would also include less relevant somatic activities. The substrate specificity for peptidase activities was detected using Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS). This highly sensitive peptidase assay utilizes tandem mass spectrometry to monitor the degradation of a synthetic peptide library. MSP-MS can simultaneously detect endo- and exo–peptidase activities, and has been successfully employed to profile the proteolytic specificities of human neutrophil extracts [61], ES products from a pathogenic fungi [62] and *S. mansoni* cercariae [63]. In addition, we employed a panel of internally quenched, fluorescent peptidyl substrates in the presence and absence of peptidase-class-specific inhibitors to identify which peptidase classes were contributing to the global activity. Overall, we characterize a number of new proteolytic activities that sets the stage for their formal identification and exploration of their respective biological functions.

2. Materials and methods

2.1. Ethics statement

Maintenance and handling of vertebrate animals were carried out in accordance with a protocol (AN107779) approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California San Francisco.

2.2. Schistosome material

A Puerto Rican isolate of *S. mansoni* is maintained in the laboratory by cycling between Golden Syrian hamsters (*Mesocricetus auratus*) and the freshwater snail, *Biomphalaria glabrata*. Female 3–5 week-old hamsters were subcutaneously injected with 200 cercariae and sacrificed 6–7 weeks post-infection using an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Adults and eggs were isolated as described [64]. Cercariae (infectious larvae) were obtained from infected snails induced to release the parasite under a light stimulus. Cercariae were chilled on ice, collected and mechanically transformed into schistosomula as described previously [65–67].

2.3. Collection of ES products

Fifty pairs of adult worms, 1000 eggs or 1000 schistosomula were washed five times in Basch medium 169 [68] supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% Fungizone (Gibco), and allowed to stand for 1 h at 37 °C in 5% CO_2. Samples were washed 10 times and then incubated at 37 °C in 5% CO_2 in the above medium supplemented with 5% fetal calf serum but in the absence of Fungizone. Adults and eggs were incubated overnight, and schistosomula were incubated for five days to allow for complete transformation from cercariae and remove contaminating cercarial peptidases. Parasite materials were washed three times in the above medium and then washed 10 times in M-199 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin, but without serum. In 5 ml of the same medium, samples were evenly distributed into a 6-well cultivation dish and incubated for 16 h at 37 °C in 5% CO_2.

Medium containing ES products was removed, filtered over an Ultrafree-MC 0.22 mm filter (Millipore), and buffer exchanged into ice-cold Dulbecco's-Phosphate-Buffered Saline (D-PBS). The medium was then concentrated to 2 ml by centrifugation at 4000 *g* and 4 °C using an Amicon 10000 Ultra-15 Centrifugal Filter Unit (Millipore). The total volume of PBS used for buffer exchange was 40 ml. Sample materials were quickly frozen in liquid nitrogen and stored at −80 °C as 100 μL aliquots. Protein concentration was measured at 280 nm on a NanoDrop 2000c (Thermo Scientific).

2.4. Multiplex peptide cleavage assay

The Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) assay was performed as previously described [63] with minor modifications. Briefly, 20 μg/ml of protein from *S. mansoni* ES products of schistosomula, adults or eggs were pre-incubated for 15 min at room temperature with 0.2% DMSO, 1 mM AEBSF (Sigma–Aldrich 76307), 100 μM E-64 (Sigma–Aldrich E-3132) or 2 mM 1,10-Phenanthroline (Sigma–Aldrich 131377) in D-PBS containing 4 mM DTT. Each reaction was split into two tubes containing an equimolar mixture of 62 peptides in D-PBS (124 total). The final assay consisted of 500 nM of each peptide, 10 μg/ml protein, 2 mM DTT, 0.1% DMSO and either 500 μM AEBSF, 50 μM E-64, 1 mM 1,10-Phenanthroline or no inhibitor in D-PBS, in a total volume of 300 μl. Aliquots were removed at defined time intervals, adjusted to <pH 3.0 with formic acid and then desalted using C18 tips (Rainin).

Mass spectrometry was performed on a LTQ FT instrument (Thermo), equipped with a nanoACUITY (Waters) ultra-performance liquid chromatography. Reverse phase LC was performed using an EZ-Spray C18 column (Thermo, ES800, PepMap, 3 μm bead size, 75 μm × 15 cm) at 600 nL/min for loading and 300 nL/min for peptide separation over a linear 65 min gradient from 2% to 30% acetonitrile in 0.1% formic acid. The mass spectrometer was operated using identical acquisition parameters as reported previously [63]. Substrate specificity profiles were generated using iceLogo software [94].

2.5. Internally quenched peptide assays

All assays were performed at room temperature in D-PBS

containing 2 mM DTT and 0.01% Triton X-100. Assays were performed in triplicate in round-bottom 96-well plates in a spectrofluorimeter (Molecular Devices Flex Station) using a λ_{ex} 328 nm and λ_{em} 393 nm. Initial velocities in relative fluorescent units per second were calculated using Softmax Pro. Protein from *S. mansoni* conditioned media was assayed with a set of internally quenched fluorescent substrates (30 μM each). The total protein concentration in the internally quenched (IQ) substrate screen was 1.95 μg/ml, 18.7 μg/ml and 3.3 μg/ml for schistosomula, adults and eggs, respectively. Each IQ substrate consisted of a 7- or 8-mer peptide flanked with 2,4-dinitrophenyl-L-lysine on the carboxyl terminus and either 7-methoxycoumarin-4-acetic acid or 7-methoxycoumarin-4-yl-acetyl-L-lysine on the amino terminus. IQ substrates were synthesized using standard Fmoc chemistry and purified to >90% by reverse phase HPLC. Inhibition assays were performed using the same concentration of proteins as above. Assays contained 30 μM of IQ substrate, 1.5% DMSO, 100 μM of E-64, 500 μM of AEBSF or 1 mM of 1,10-Phenanthroline.

3. Results

3.1. Detection of cleavage sites derived from peptidases in S. mansoni ES products

We took a global and unbiased approach to characterizing the proteolytic components of the ES products of three *S. mansoni* life-stages that parasitize the mammalian host, namely schistosomula, adults and eggs. After extensive washing to remove blood and serum components, including contaminant proteases, each developmental stage was placed in serum-free medium overnight. The ES products (conditioned medium) were then concentrated and added to a mixture of 124 physicochemically diverse peptides that are each 14-residues in length. Cleavage of any one of the 1612 available peptide bonds within these peptides can be readily detected by LC-MS/MS sequencing. This assay is termed Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) and has been previously used with *S. mansoni* to characterize the specificity of peptidases in conditioned water from parasite-infected snails [63].

Assays on *S. mansoni* ES products were performed at pH 7.4 to mimic physiological pH of the mammalian host. Incubation of schistosomula, adults and eggs ES peptidases with the peptide library for 15 min resulted in 23, 7 and 15 cleavage sites, respectively (Fig. 1A). However, after the longest incubation of 1200 min, the number of cleavage sites increased to 107, 151 and 301, respectively. In addition, cleavage sites were assessed at the intermediate time intervals of 60 and 240 min (Supplementary File 1).

The complexity of these hydrolytic events is illustrated using a sample peptide, *AYNnWSLYRnlRQE*, from which multiple cleavage sites were detected at various time intervals (Fig. 1B). Peptidases secreted from schistosomula, cleave at the Trp–Ser and Arg-Nle site and products of this hydrolysis were evident after only 15 min. After 60 min, additional cleavage products were detected that corresponded to hydrolysis at the Tyr–Arg bond. Unlike the other two stages, schistosomula ES peptidases could not cleave the Nle-Ile bond. Adult peptidase activity also cleaved the Trp–Ser and Arg-Nle bonds but these products appeared at later time intervals compared to hydrolysis of the Nle-Ile bond. No cleavage of the Tyr–Arg bond was evident in the adult ES products even after 1200 min incubation, however, products derived from Tyr–Asn hydrolysis were found after 240 min incubation. Finally, ES peptidases from eggs preferentially cleaved at the Arg-Nle site within 15 min and later at the Trp–Ser and Nle-Ile sites. Like adults, egg ES peptidases did not cleave the Tyr–Arg bond. Thus, interrogation of just this single sample peptide from the mixture of 124, indicates that different peptidases are present in the ES products of each of the *S. mansoni* intra-mammalian stages.

The presence of different peptidases in the conditioned media from each of these developmental stages was investigated by directly comparing the cleavage sites. For this, the 145 sites identified after 240 min in the egg ES products was compared to the 107 and 151 sites identified from 1200 min incubation of schistosomula and adults peptidases, respectively. These assays correspond to the earliest incubation time required to digest at least 5% of the 1612 peptide bonds in the library (≥81 cleavage sites). Peptidases from schistosomula, adults and eggs cleaved at 35, 79 and 62 unique sites, respectively, whereas 45 sites were common to all three (Fig. 1C). This analysis confirms that unique peptidases are present in the ES products from each of the intra-mammalian life stages.

In our experience, exopeptidases that remove mono-, di- or tri-peptides from the amino or carboxy terminus of proteins and oligopeptides are difficult to detect with standard reporter substrates due to the positioning of the fluorescent or colorimetric reporter group. For carboxypeptidases, the reporter group blocks the carboxyl terminus and therefore prevents cleavage. For aminopeptidases, substrates that are too long or too short, will not be hydrolyzed correctly between the canonical P1 residue and the reporter group. Our MSP-MS approach can simultaneously detect exo- and endo-peptidase activity because the peptides employed have free amino and carboxyl termini. The location of each cleavage site within the 14-mer peptides was compared for egg ES peptidases after 240 min incubation and adult and schistosomula ES peptidases after 1200 min incubation (Fig. 1D). In general, the majority of peptide bond hydrolysis occurred away from the termini indicating that endopeptidases are most active. However, enzymes in the adult ES products cleaved between position 2 and 3 of the 14-mer peptides at a higher frequency than egg and schistosomula peptidases indicating that an enzyme with di-aminopeptidase activity may be present. Conversely, there is a higher frequency of cleavage by egg ES products between position 12 and 13 which may represent a di-carboxypeptidase activity.

3.2. Comparison of the substrate specificity profiles between life cycle stages

For each of the cleavage sites generated by the ES peptidases, we obtained prime and non-prime site substrate specificity information (Fig. 2). Using iceLogo software, a P4 to P4′ substrate signature was generated for all cleavage sites that occur after a defined incubation time. Peptidases secreted by schistosomula preferentially degraded peptides on the C-terminal side of Arg or Lys residues. This type of substrate specificity is commonly called “trypsin-like”. Gln was most often found in the P2 position and Ser or Arg were frequently found at P1′. In addition, cleavage rarely or never occurred at the C-terminal side of Gly, Pro, Nle or Glu or at the N-terminal side of Pro or Asn (Fig. 2A).

In contrast to schistosomula, much greater cleavage promiscuity was apparent in the ES products of adults suggesting that there are multiple enzymes present (Fig. 2B).Adult peptidases also preferentially cleaved at sites when Arg or Trp were present in the P1 position and Ser or Arg at P1′. The degradation of peptides by the egg ES peptidases had a preference for Arg and Gln at P1, Arg at P1′ and bulky hydrophobic residues at P3 (Fig. 2C).

To complete the life cycle, we included the substrate signature generated from our previous study using water conditioned for 1200 min with *S. mansoni* infected and non-infected *B. glabrata* snails [63]. Non-infected snails secrete at least one peptidase with a trypsin-like specificity (Arg and Lys at P1; Fig. 2D). This is consistent with the previous biochemical characterization of a major tryptase enzyme in snail extracts [69]. The substrate signature generated from conditioned water from *S. mansoni* infected-snails presented a

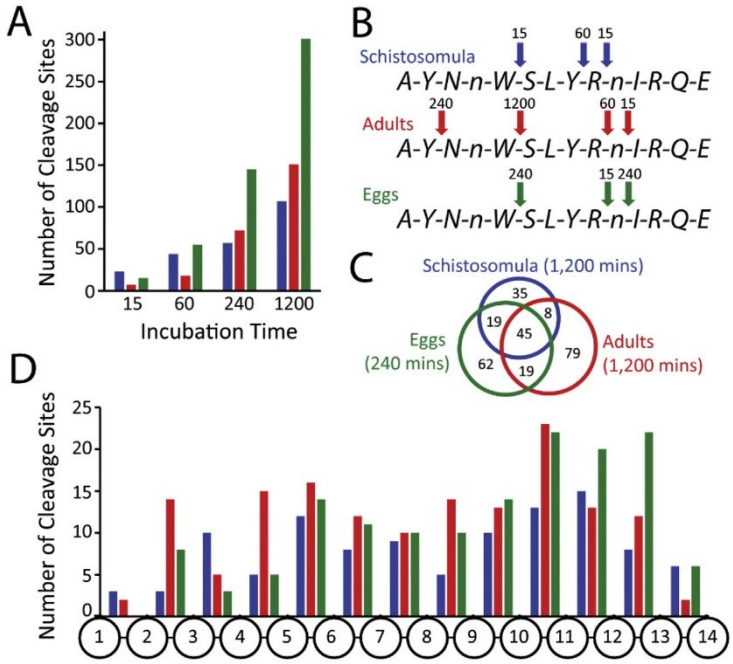

Fig. 1. Detection of proteolytic activity in the ES products of *S. mansoni*. A. ES products from schistosomula (blue), adult worms (red) and eggs (green) were incubated for 15 to 1200 min with a 14-mer peptide library. LC-MS/MS sequencing was used to detect the appearance of peptidase cleavage sites B. A sample 14-mer peptide illustrating the complexity of peptidase cleavage. The position and time (minutes) that cleavage products were first detected are indicated. Amino acids are shown in single letter code. Lowercase "n" corresponds to norleucine (Nle). C. Venn Diagram showing the number of unique and shared cleavage sites. D. Spatial distribution of cleavage sites within the 14-mer peptide scaffold.

different substrate specificity profile (Fig. 2E). Infected snails in water release cercariae which contain a number of chymotrypsin-like serine peptidases known collectively as 'cercarial elastase'. An in-depth characterization of these enzymes uncovered a P1 specificity for Phe and Tyr (Fig 2E). In addition, a preference for Pro at P2 is recorded and this is consistent with the known specificity of purified cercarial elastase using P1–P4 substrate positional scanning [70]. Schistosomula are generated from cercariae by mechanical shearing of tails followed by *in vitro* culture [65]. After 5 days of culture *in vitro*, it is clear that the chymotrypsin-like hydrolysis signature of the cercarial elastase is replaced by one that is trypsin-like.

3.3. Detection of peptidase activity in ES products using IQ substrates

As the majority of activity in ES products was derived from endopeptidases, we utilized a panel of internally quenched (IQ) fluorescent peptides with diverse sequences to quantify this activity. These substrates were previously synthesized by our group to detect aspartic acid and glutamic acid [71], cysteine [72] and serine [73] peptidases from a variety of microbial sources. These peptides were collectively used to detect proteolytic activity in conditioned media from the fungal pathogen, *Pseudogymnoascus destructans* [62]. Each IQ peptide is either seven or eight amino acids long and flanked on the N-terminus by a fluorophore and on the C-terminus by a fluorescent quenching group. Cleavage of any bond results in an increase in fluorescence due to the separation of the quencher from the fluorophore.

Peptidases in the ES products from each life cycle stage hydrolyzed three IQ substrates containing the sequences QCACSNHE, tQASSRS and GRFGVWKA (Fig. 3). No other peptides were cleaved by schistosomula ES products. Egg-conditioned medium cleaved 5 additional IQ substrates, and in general showed a higher specific activity relative to schistosomula and adult enzymes. This is consistent with egg peptidases cleaving at a greater number of sites in the MSP-MS assay. The conditioned medium from adults cleaved all IQ substrates but generally had low specific activity relative to the schistosomula and egg ES enzymes. tQASSRS was the commonly cleaved IQ substrate, and was therefore deemed to be a useful reporter substrate to quantitatively measure proteolytic activity in the presence and absence of class-specific inhibitors.

3.4. Investigating the contribution of serine, cysteine and metallo-peptidases using class-specific inhibitors

Using the tQASSRS substrate, ES products from each of the *S. mansoni* developmental stages were treated with class-specific inhibitors that target serine, cysteine and metallo-peptidases. All assays were performed at pH 7.4, at which aspartic acid peptidases are unlikely to be active. Therefore, pepstatin A that targets aspartic acid peptidases was not included in the inhibitor screen.

3.4.1. Schistosomula

Cleavage of tQASSRS was unchanged upon treatment with the metallopeptidase inhibitor 1,10-Phenanthroline when compared to the non-inhibited control (DMSO) while the cysteine peptidase inhibitor E-64 actually increased activity. However, in the presence of the serine peptidase inhibitor, AEBSF, turnover of the fluorescent

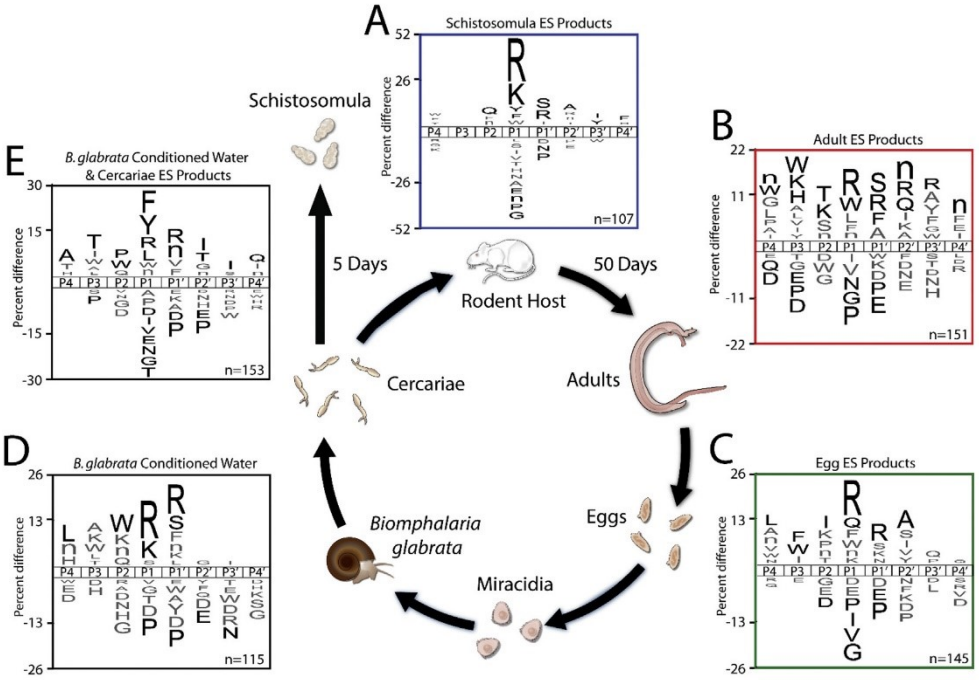

Fig. 2. Generation of a proteolytic specificity signature in the ES products of *S. mansoni* at different life cycle stages. Generation of a substrate specificity iceLogo signature for A. schistosomula, B. adult and C. egg secretions following a 240 or 1200 min incubation period with the 14-mer peptide library. Amino acids colored black are significantly increased ($p < 0.05$) in the position relative to a control dataset that corresponds to all possible cleavage sites. Amino acids above the X-axis are found at the given position with higher frequency than the control dataset while amino acids below the axis are rarely or never found at the given position. IceLogo signature showing the substrate specificity profiles of conditioned water from D. uninfected and E. *S. mansoni* infected *Biomphalaria glabrata*.

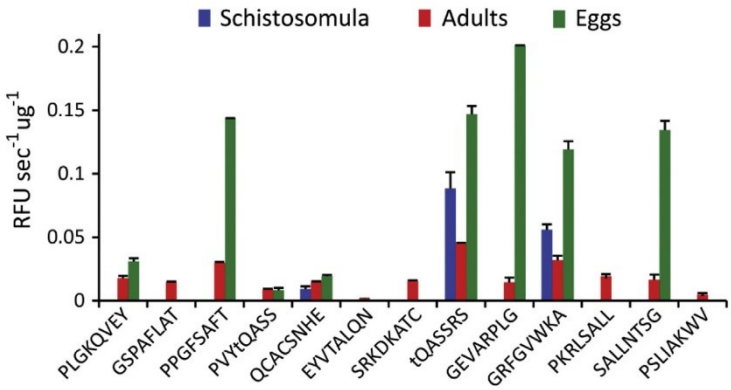

Fig. 3. Quantitation of proteolytic activity in ES products of *S. mansoni* at different life cycle stages. Proteolytic activity in the conditioned media was detected using internally quenched fluorescent substrates. Amino acids are described in single letter code and lowercase 't' corresponds to *tert*-butyl glycine.

substrate was abolished (Fig. 4A). These data are consistent with the presence of a predominant serine peptidase activity.

MSP-MS assays were performed with the same inhibitor treated ES products that were used in the IQ studies. Using the sample 14-mer peptide highlighted in Fig. 1B, we show that treatment of medium with E-64 results in a cleavage pattern that is identical to DMSO-treated control (Fig. 4B). In contrast, AEBSF prevented hydrolysis of the Trp–Ser and Tyr–Arg bonds and greatly delayed hydrolysis of the Arg-Nle bond, which was only recorded after 1200 min. Two new cleavage sites (Tyr–Asn and Nle-Ile) appeared after 1200 min that were never observed in the DMSO-treated control (Fig. 4B). The appearance of 'new' cleavage sites following treatment with an inhibitor has been observed previously [62]. These sites were not detected in the DMSO-treated control assay because the peptide was rapidly degraded into tri-, tetra- and pepta-peptides by the serine peptidase(s) and therefore any subsequent cleavage sites would not be readily detected by mass spectrometry. Finally, treatment with 1,10-Phenanthroline caused a delay in the processing of the Tyr–Arg bond from 60 to 240 min but otherwise the cleavage sites remained unchanged.

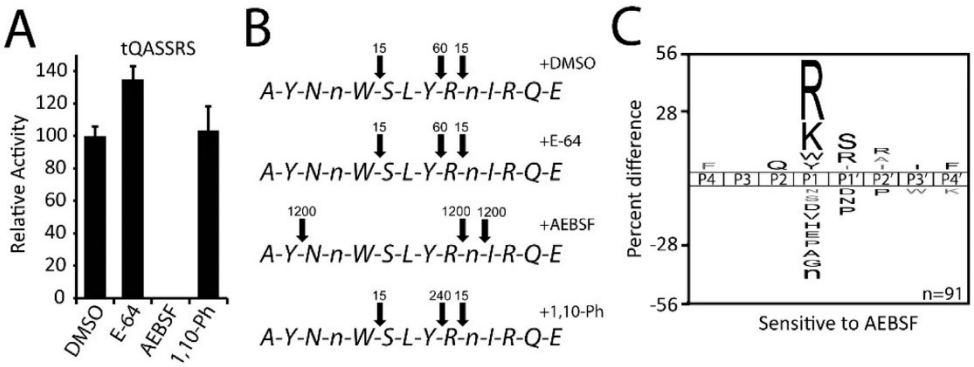

Fig. 4. Use of class-specific inhibitors to characterize peptidase activity and specificity in schistosomula secretion. A. ES products were assayed with the internally quenched tQASSRS substrate in the presence of class-specific inhibitors. B. Use of a sample 14-mer peptide to illustrate the time dependent changes in cleavage site accumulation in the presence of class-specific inhibitors compared to a non-inhibited DMSO control. C. An iceLogo substrate profile generated from all cleavage sites in the 14-mer peptide library that were sensitive to AEBSF.

Treatment of schistosomula ES products with AEBSF resulted in the delay in appearance of 91 of the 107 cleavage sites that were detected in the DMSO-treated control. These sites were termed "sensitive to AEBSF" and frequently had Arg and Lys in the P1 position (Fig. 4C). Based on this substrate profile it is reasonable to suggest that a single major peptidase with trypsin-type specificity is present in the ES products of schistosomula.

3.4.2. Adults

Treatment of adult ES products with either E-64 or AEBSF resulted in a 19% and 35% decrease in the overall cleavage of tQASSRS, respectively (Fig. 5A). However, when these compounds were combined, E-64 provided no additional inhibition, indicating that this inhibitor may be targeting the serine peptidase(s). Competitive inhibition of bovine trypsin by E-64 has been previously reported [74]. 1,10-Phenanthroline decreased activity by more than 80%, indicating that metallo-peptidases predominate in adult ES products under the conditions employed. Combining 1,10-Phenanthroline with E-64 and AEBSF completely inhibited cleavage of the tQASSRS substrate (Fig. 5A).

When AEBSF was added to the MSP-MS assay and compared to a DMSO-treated control, 59 of 151 cleavage sites were sensitive to this inhibitor (Fig. 5B). The specificity profile of the AEBSF-sensitive enzymes had a preference for Arg at P1, but a much lower preference for Lys compared to the schistosomula ES peptidase. This indicates that it is unlikely that the same serine peptidases are being measured in ES products of schistosomula and adults.

When the adult ES products were treated with 1,10-Phenanthroline, 107 of the total of 151 cleavage sites in the 14-mer peptide library were sensitive to this agent (Fig. 5C). The substrate specificity profile of the 1,10-Phenanthroline-sensitive enzymes had a preference for bulky residues at P3, Leu at P1 and Arg, Phe and Ser at P1′. In addition, Gln and Lys were most often found at P2′ whereas Arg, Ala and Tyr were frequently identified at P3′.

Interestingly, all cleavage sites that occurred between the 2nd and 3rd position of each 14-mer peptide were sensitive to 1,10-Phenanthroline but only two were sensitive to AEBSF (Fig. 5D). In some cases, AEBSF treatment resulted in an earlier appearance of the cleavage product, when compared to the DMSO control. For example, removal of AY from the sample tetradacapeptide, *AYNnWSLYRnIRQE* occurs after 240 min incubation in the DMSO-treated assay, but the dodecapeptide cleavage product can be detected after only 15 min incubation in the AEBSF treated assay. Thus, in addition to the metallo–endopeptidase activity sensitive to 1,10-Phenanthroline, there is a least one other metallo-peptidase in the ES products with a specificity for amino-terminal dipeptides.

3.4.3. Eggs

Treatment of egg ES products with AEBSF completely inhibited cleavage of tQASSRS indicating that serine peptidase activity is a major proteolytic component (Fig. 6A). Cysteine and metallo-peptidases are also present as E-64 and 1,10-Phenanthroline decreased activity by 61% and 15%, respectively. A substrate specificity profile was generated for both the AEBSF-sensitive (Fig. 6B) and E-64-sensitive (Fig. 6C) cleavage sites. Cleavage sites that were sensitive to AEBSF had a trypsin-like substrate specificity profile using the MSP-MS assay. Interestingly, the cleavage sites that were sensitive to E-64 also had a strong P1 preference for Arg and Gln and generally accepted hydrophobic residues at P2, consistent with that known for cysteine cathepsins [75–77].

The MSP-MS assay on the egg ES products indicated that not all of the cleavage sites were sensitive to either AEBSF or E-64 treatment. Indeed, 61 of the original 145 cleavages identified in the control-treated assay were resistant to both inhibitors suggesting that enzymes other than serine or cysteine peptidases are also present (Fig. 6D). To further probe the egg-conditioned media we utilized the IQ substrate, GRFGVWKA, identified in Fig. 3. This substrate was also cleaved by peptidases in the conditioned media from all developmental stages tested, although with lower activity than the tQASSRS substrate. Using the GRFGVWKA substrate, AEBSF treatment only caused a 35% decrease in overall activity, whereas 1,10-Phenanthroline abolished activity (Fig. 6E). Using these same conditions, the 1,10-Phenanthroline-treated ES products were interrogated with the MSP-MS assay. The resulting sensitive cleavage sites had a preference for Trp, Ile, Ser and Arg at P4, P3, P2 and P1, respectively. In addition, a P1′ preference for Arg was evident. Notably, this 1,10-Phenathroline sensitive peptidase differs in specificity from the metallo-peptidases in adults. Taken together, these studies indicate that serine, cysteine and metallo-peptidases are all active in the ES products of *S. mansoni* eggs.

4. Discussion

Understanding the biological function of a peptidase in any organism, including *Schistosoma*, requires orthogonal data inputs, including gene expression, protein localization, post-translational modifications and substrate specificity. Global 'omic' analyses

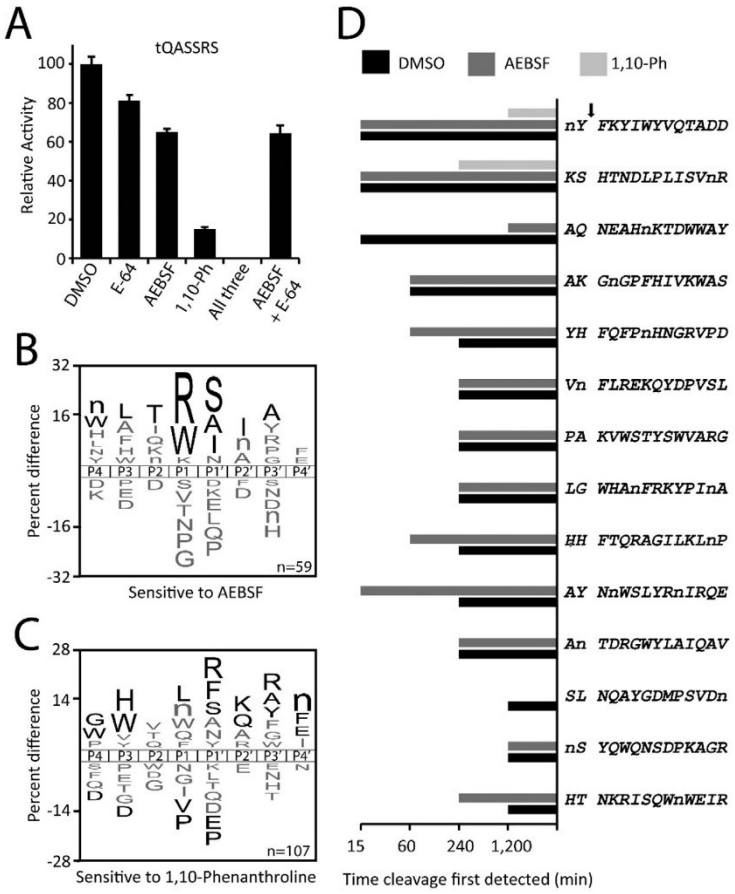

Fig. 5. Use of class-specific inhibitors to characterize peptidase activity and specificity in adult secretion. A. ES products were assayed with the internally quenched tQASSRS substrate in the presence of class-specific inhibitors. B. An iceLogo substrate profile consisting of all cleavage sites in 14-mer peptides that were sensitive to AEBSF treatment. C. An iceLogo substrate profile consisting of all cleavage sites in 14-mer peptides that were sensitive to 1,10-Phenanthroline treatment. D. Analysis of the di-aminopeptidase specificity detected in adult ES products. The bar graph represents the time interval at which each the cleavage product was first detected.

such as those have made key contributions in these respects. To date, however, there has been no global *functional* profiling (qualitative nor quantitative) of the peptidases across developmental stages. Our employment of peptidase-agnostic technologies in combination with class-specific peptidase inhibitors reveals previously unrecognized activities that are released by key schistosome developmental stages that parasitize the human host. The technologies applied to schistosomula, adults and eggs have been previously utilized in a number of different contexts [61,62] including to characterize *S. mansoni* cercarial secretions [63]. The present report is, therefore, a natural extension of that work. We employed ES products as these are likely to contain peptidases operating at the host parasite interface and that potentially influence host immunology and physiology. Finally, ES products were collected and processed at neutral (physiologic) pH to inactivate or at least mitigate the proteolytic contributions of aspartic and cysteine proteases [39,78,79], not least those adult gut proteases that have been extensively characterized [11,13,29,80,81] and are regurgitated by the worm into culture medium [11,78,82,83]. Thus, our conditions facilitate the identification of novel peptidolytic activities, specifically, metallo- and serine proteases.

We utilized two IQ substrates as reporters of activity and inhibitor sensitivity. The cleavage sites within these substrates were not investigated as the specificity profile was revealed in the subsequent MSP-MS assays. The proteolytic profile of schistosomular ES products is the simplest measured and centers on a single or predominant serine peptidase with a strict P1− P1′ specificity for charged amino acids and only minor amino acid engagements at the other prime and non-prime binding sites. The activity may be due to one or more of a number of schistosomular serine peptidases we previously measured by both gene expression profiling and functional activity analyses with small peptidyl substrates [19]. Importantly, the specificity profile of this serine peptidase activity differs markedly from the chymotrypsin-like cercarial elastase that is secreted by the *S. mansoni* cercariae and from which we derive schistosomula [63,70].

In contrast to schistosomula, the specificity profiles of adults and eggs are much more complex and different from one another with respect to the proteolytic contributions of serine, metallo- and cysteine proteases. Adults produce a serine peptidase that has a strong preference for P1-Arg but not Lys. Generally, trypsin-like serine peptidases cleave peptides with these P1-amino acids equally well. These data support our previous finding that a number of different trypsin peptidases are expressed in adult *S. mansoni*

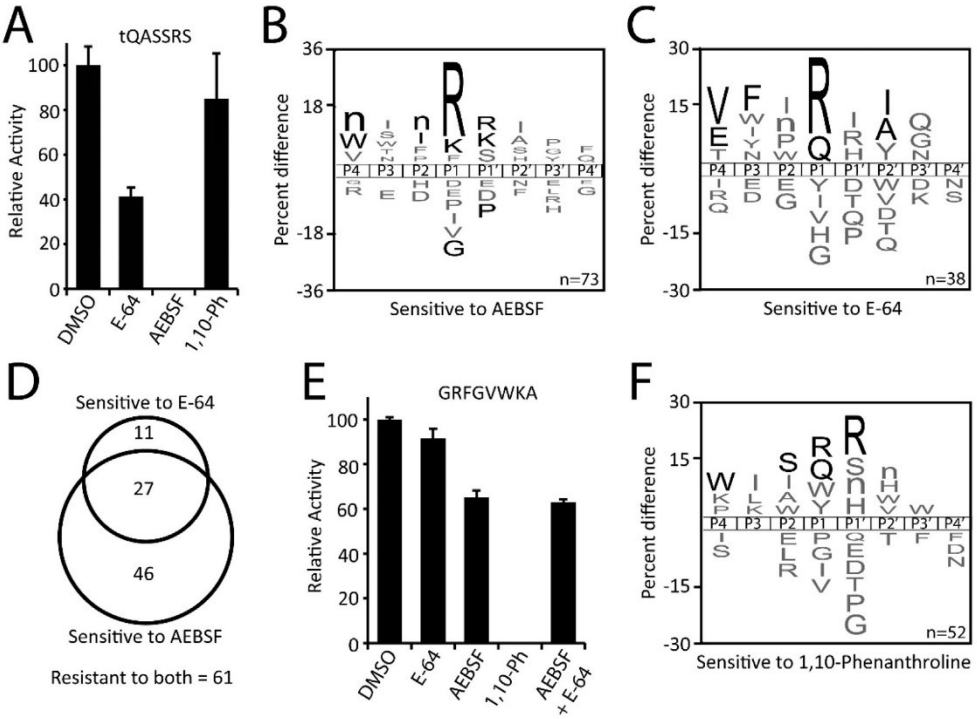

Fig. 6. Use of class-specific inhibitors to characterize peptidase activity and specificity in egg secretion A. ES products were assayed with the internally quenched tQASSRS substrate in the presence of class-specific inhibitors. B. An iceLogo substrate profile consisting of all cleavage sites in the 14-mer peptides that were sensitive to AEBSF treatment. C. An iceLogo substrate profile consisting of all cleavage sites in the 14-mer peptides that were sensitive to E-64 treatment. D. A Venn diagram showing that many of the cleavage sites are resistant to both AEBSF and E-64. E. ES products were assayed with the internally quenched GRFGVWKA substrate in the presence of class-specific inhibitors. F. An iceLogo substrate profile consisting of all cleavage sites in the 14-mer peptides that were sensitive to 1,10-Phenanthroline treatment.

[19]. In addition, a second serine peptidase activity is present that has chymotrypsin-like P1 specificity for Trp. However, this enzyme has little preference for P1-Tyr, -Phe and -Leu which are normally associated with chymotrypsin. Adults also produce at least two metallo-peptidases, one of which is a metallo di-aminopeptidase activity, thus underscoring the utility of the MSP-MS approach to not only detect endopeptidase activity but also exopeptidase activity. Neither of the metallo-peptidase specificity profiles identified are similar to those recorded for a M17 family leucine aminopeptidase activity previously characterized in *S. mansoni* [84,85].

Like adult ES products, those from eggs contain serine and metallo-peptidase activity profiles, however, these differ from the adult profiles and from each other suggesting that different enzymes are being measured. It's possible that the serine peptidase activity may be in part due to a PMSF-sensitive fibrinolytic activity previously described in egg extracts [18] although no protein identification for this activity was subsequently carried out. Regarding the metallo–endopeptidase activity, we are not aware of such being described in egg ES products but it's clear that a major 1,10-Phenanthroline-sensitive activity is present. Interestingly, egg conditioned medium also contains a cysteine protease activity that is robustly active under the neutral pH conditions employed. This is in contrast to adult ES products that predominantly contain neutral pH-labile cathepsins that arise from the gastrointestinal tract [78]. The presence of cysteine protease activity has been described in eggs [86,87] and miracidia (which eventually emerge from the egg) [88]. Peptidase activity has also been measured at neutral pH in egg ES products [89] and live eggs were shown to degrade the glycoprotein component of an artificial extracellular matrix at neutral pH. This activity was enhanced in the presence of reducing agent suggesting a role for cysteine peptidases [90]. Accordingly, it's possible that eggs release a specialized cathepsin–like activity that facilitates their passage through host tissues and their eventual escape into the environment via the urogenital or digestive tracts.

Our application of peptidase-agnostic technologies in combination with peptidase class-specific inhibitors reveals the heretofore unrecognized complexity of peptidolytic activities released by key schistosome life-stages parasitizing the human host. Further biochemical studies are required to identify the peptidases responsible but there are now well-annotated genomic and transcriptomic data (references cited in the Introduction) that can be brought to bear in such studies. Aided by knowledge of both substrate specificity and peptidase class, we can now develop tools to characterize the individual enzymes in greater detail, *e.g.*, activity-based probes that bind to the peptidase active site. Such probes require knowledge of both the substrate recognition sequence and the nature of the active-site nucleophile, and have been previously engineered to identify and characterize peptidases, and image disease as biomarkers [91–93].

5. Conclusion

This study describes the application of an unbiased and global technology to characterize *S. mansoni* excretion-secretion peptidase activity. Each intra-mammalian developmental stage

produces a different set of activities and all stages except cercariae secrete a trypsin-type serine peptidase. In addition, a metallopeptidase with dipeptidase specificity was observed in adult ES products and eggs release a cysteine protease that is active at neutral pH. These studies will facilitate the development of selective active-site directed affinity probes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2015.09.025.

References

[1] P. Steinmann, J. Keiser, R. Bos, M. Tanner, J. Utzinger, Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk, Lancet Infect. Dis. 6 (2006) 411–425.
[2] C.H. King, M. Dangerfield-Cha, The unacknowledged impact of chronic schistosomiasis, Chronic Illn. 4 (2008) 65–79.
[3] S.C. Hall, E.L. Kehoe, Case reports. Prolonged survival of *Schistosoma japonicum*, Calif. Med. 113 (1970) 75–77.
[4] E.S. Loker, A comparative study of the life-histories of mammalian schistosomes, Parasitology 87 (Pt 2) (1983) 343–369.
[5] K.S. Warren, Schistosomiasis: host-pathogen biology, Rev. Infect. Dis. 4 (1982) 771–775.
[6] B. Gryseels, K. Polman, J. Clerinx, L. Kestens, Human schistosomiasis, Lancet 368 (2006) 1106–1118.
[7] D.G. Colley, A.L. Bustinduy, W.E. Secor, C.H. King, Human schistosomiasis, Lancet 383 (2014) 2253–2264.
[8] J. Utzinger, K. N'Goran E, C.R. Caffrey, J. Keiser, From innovation to application: socialecological context, diagnostics, drugs and integrated control of schistosomiasis, Acta Trop. 120 (2011) S121–S137.
[9] N.D. Rawlings, M. Waller, A.J. Barrett, A. Bateman, MEROPS: the database of proteolytic enzymes, their substrates and inhibitors, Nucleic Acids Res. 42 (2014) D503–D509.
[10] K. Brix, W. Stöcker (Eds.), Proteases: Structure and Function, Spinger Verlag, Vieena, 2013.
[11] C.R. Caffrey, J.H. McKerrow, J.P. Salter, M. Sajid, Blood 'n' guts: an update on schistosome digestive peptidases, Trends Parasitol. 20 (2004) 241–248.
[12] M.E. Morales, G. Rinaldi, G.N. Gobert, K.J. Kines, J.F. Tort, P.J. Brindley, RNA interference of *Schistosoma mansoni* cathepsin D, the apical enzyme of the hemoglobin proteolysis cascade, Mol. Biochem. Parasitol. 157 (2008) 160–168.
[13] M. Kasny, L. Mikes, V. Hampl, J. Dvorak, C.R. Caffrey, J.P. Dalton, P. Horak, Chapter 4. Peptidases of trematodes, Adv. Parasitol. 69 (2009) 205–297.
[14] J.H. McKerrow, C. Caffrey, B. Kelly, P. Loke, M. Sajid, Proteases in parasitic diseases, Annu. Rev. Pathol. 1 (2006) 497–536.
[15] W.S. Carvalho, C.T. Lopes, L. Juliano, P.M. Coelho, J.R. Cunha-Melo, W.T. Beraldo, J.L. Pesquero, Purification and partial characterization of kininogenase activity from *Schistosoma mansoni* adult worms, Parasitology 117 (Pt 4) (1998) 311–319.
[16] C. Cocude, C. Pierrot, C. Cetre, J. Fontaine, C. Godin, A. Capron, J. Khalife, Identification of a developmentally regulated *Schistosoma mansoni* serine protease homologous to mouse plasma kallikrein and human factor I, Parasitology 118 (Pt 4) (1999) 389–396.
[17] C. Cocude, C. Pierrot, C. Cetre, C. Godin, A. Capron, J. Khalife, Molecular characterization of a partial sequence encoding a novel *Schistosoma mansoni* serine protease, Parasitology 115 (Pt 4) (1997) 395–402.
[18] M.J. Doenhoff, R.G. Stanley, D. Pryce, R.C. Curtis, H. Parry, K. Griffiths, C.L. Jackson, Identification of a fibrinolytic enzyme in *Schistosoma mansoni* eggs and modulated blood fibrinogen metabolism in *S. mansoni*-infected mice, Parasitology 126 (2003) 231–234.
[19] M. Horn, P. Fajtova, L. Rojo Arreola, L. Ulrychova, P. Bartosova-Sojkova, Z. Franta, A.V. Protasio, D. Opavsky, J. Vondrasek, J.H. McKerrow, M. Mares, C.R. Caffrey, J. Dvorak, Trypsin- and chymotrypsin-like serine proteases in *Schistosoma mansoni*– 'the undiscovered country', PLoS Negl. Trop. Dis. 8 (2014) e2766.
[20] L.A. de Oliveira Fraga, E.W. Lamb, E.C. Moreno, M. Chatterjee, J. Dvorak, M. Delcroix, M. Sajid, C.R. Caffrey, S.J. Davies, Rapid induction of IgE responses to a worm cysteine protease during murine pre-patent schistosome infection, BMC Immunol. 11 (2010) 56.
[21] B.C. Figueiredo, N.D. Ricci, N.R. de Assis, S.B. de Morais, C.T. Fonseca, S.C. Oliveira, Kicking in the guts: *Schistosoma mansoni* digestive tract proteins are potential candidates for vaccine development, Front. Immunol. 6 (2015) 22.
[22] H. Tallima, J.P. Dalton, R. El Ridi, Induction of protective immune responses against *Schistosomiasis haematobium* in hamsters and mice using cysteine peptidase-based vaccine, Front. Immunol. 6 (2015) 130.
[23] J.Y. Gauthier, N. Chauret, W. Cromlish, S. Desmarais, T. Duong le, J.P. Falgueyret, D.B. Kimmel, S. Lamontagne, S. Leger, T. LeRiche, C.S. Li, F. Masse, D.J. McKay, D.A. Nicoll-Griffith, R.M. Oballa, J.T. Palmer, M.D. Percival, D. Riendeau, J. Robichaud, G.A. Rodan, S.B. Rodan, C. Seto, M. Therien, V.L. Truong, M.C. Venuti, G. Wesolowski, R.N. Young, R. Zamboni, W.C. Black, The discovery of odanacatib (MK-0822), a selective inhibitor of cathepsin K, Bioorg Med. Chem. Lett. 18 (2008) 923–928.
[24] O. Barauskas, A.C. Corsa, R. Wang, S. Hluhanich, D. Jin, M. Hung, H. Yang, W.E.t. Delaney, B.E. Schultz, Binding kinetics, potency, and selectivity of the hepatitis C virus NS3 protease inhibitors GS-9256 and vedroprevir, Biochim. Biophys. Acta 1840 (2014) 3292–3298.
[25] U. Schlomann, G. Koller, C. Conrad, T. Ferdous, P. Golfi, A.M. Garcia, S. Hofling, M. Parsons, P. Costa, R. Soper, M. Bossard, T. Hagemann, R. Roshani, N. Sewald, R.R. Ketchem, M.L. Moss, F.H. Rasmussen, M.A. Miller, D.A. Lauffenburger, D.A. Tuveson, C. Nimsky, J.W. Bartsch, ADAM8 as a drug target in pancreatic cancer, Nat. Commun. 6 (2015) 6175.
[26] M.M. Wasilewski, K.C. Lim, J. Phillips, J.H. McKerrow, Cysteine protease inhibitors block schistosome hemoglobin degradation in vitro and decrease worm burden and egg production in vivo, Mol. Biochem. Parasitol. 81 (1996) 179–189.
[27] M.H. Abdulla, D.S. Ruelas, B. Wolff, J. Snedecor, K.C. Lim, F. Xu, A.R. Renslo, J. Williams, J.H. McKerrow, C.R. Caffrey, Drug discovery for schistosomiasis: hit and lead compounds identified in a library of known drugs by medium-throughput phenotypic screening, PLoS Negl. Trop. Dis. 3 (2009) e478.
[28] A. Farid, A.A. Malek, I. Rabie, A. Helmy, A.M. El Amir, Overview on cysteine protease inhibitors as chemotherapy for Schistosomiasis mansoni in mice and also its effect on the parasitological and immunological profile, Pak. J. Biol. Sci. PJBS 16 (2013) 1849–1861.
[29] A. Jilkova, P. Rezacova, M. Lepsik, M. Horn, J. Vachova, J. Fanfrlik, J. Brynda, J.H. McKerrow, C.R. Caffrey, M. Mares, Structural basis for inhibition of the cathepsin B drug target from the human blood fluke, *Schistosoma mansoni*, J. Biol. Chem. 286 (2011) 35770–35781.
[30] M. Sajid, J.H. McKerrow, Cysteine proteases of parasitic organisms, Mol. Biochem. Parasitol. 120 (2002) 1–21.
[31] M. Marco, J.M. Coteron, Falcipain inhibition as a promising antimalarial target, Curr. Top. Med. Chem. 12 (2012) 408–444.
[32] N. Drinkwater, R.S. Bamert, K.K. Sivaraman, A. Paiardini, S. McGowan, X-ray crystal structures of an orally available aminopeptidase inhibitor, Tosedostat, bound to anti-malarial drug targets PfA-M1 and PfA-M17, Proteins 83 (2015) 789–795.
[33] A.A. Rascon Jr., J.H. McKerrow, Synthetic and natural protease inhibitors provide insights into parasite development, virulence and pathogenesis, Curr. Med. Chem. 20 (2013) 3078–3102.
[34] M. Sajid, S.A. Robertson, L.S. Brinen, J.H. McKerrow, Cruzain : the path from target validation to the clinic, Adv. Exp. Med. Biol. 712 (2011) 100–115.
[35] J.J. Vermeire, L.D. Lantz, C.R. Caffrey, Cure of hookworm infection with a cysteine protease inhibitor, PLoS Negl. Trop. Dis. 6 (2012) e1680.
[36] M. Ndao, M. Nath-Chowdhury, M. Sajid, V. Marcus, S.T. Mashiyama, J. Sakanari, E. Chow, Z. Mackey, K.M. Land, M.P. Jacobson, C. Kalyanaraman, J.H. McKerrow, M.J. Arrowood, C.R. Caffrey, A cysteine protease inhibitor rescues mice from a lethal *Cryptosporidium parvum* infection, Antimicrob. Agents Chemother. 57 (2013) 6063–6073.
[37] A. Jilkova, M. Horn, P. Rezacova, L. Maresova, P. Fajtova, J. Brynda, J. Vondrasek, J.H. McKerrow, C.R. Caffrey, M. Mares, Activation route of the *Schistosoma mansoni* cathepsin B1 drug target: structural map with a glycosaminoglycan switch, Structure 22 (2014) 1786–1798.
[38] A. Ricciardi, J.P. Dalton, M. Ndao, Evaluation of the immune response and protective efficacy of *Schistosoma mansoni* cathepsin B in mice using CpG dinucleotides as adjuvant, Vaccine 33 (2015) 346–353.
[39] C.P. Brady, A.J. Dowd, P.J. Brindley, T. Ryan, S.R. Day, J.P. Dalton, Recombinant expression and localization of *Schistosoma mansoni* cathepsin L1 support its

role in the degradation of host hemoglobin, Infect. Immun. 67 (1999) 368–374.
[40] C.P. Brady, P.J. Brindley, A.J. Dowd, J.P. Dalton, *Schistosoma mansoni*: differential expression of cathepsins L1 and L2 suggests discrete biological functions for each enzyme, Exp. Parasitol. 94 (2000) 75–83.
[41] M. Sajid, J.H. McKerrow, E. Hansell, M.A. Mathieu, K.D. Lucas, I. Hsieh, D. Greenbaum, M. Bogyo, J.P. Salter, K.C. Lim, C. Franklin, J.H. Kim, C.R. Caffrey, Functional expression and characterization of *Schistosoma mansoni* cathepsin B and its trans-activation by an endogenous asparaginyl endopeptidase, Mol. Biochem. Parasitol. 131 (2003) 65–75.
[42] M. Berriman, B.J. Haas, P.T. LoVerde, R.A. Wilson, G.P. Dillon, G.C. Cerqueira, S.T. Mashiyama, B. Al-Lazikani, L.F. Andrade, P.D. Ashton, M.A. Aslett, D.C. Bartholomeu, G. Blandin, C.R. Caffrey, A. Coghlan, R. Coulson, T.A. Day, A. Delcher, R. DeMarco, A. Djikeng, T. Eyre, J.A. Gamble, E. Ghedin, Y. Gu, C. Hertz-Fowler, H. Hirai, Y. Hirai, R. Houston, A. Ivens, D.A. Johnston, D. Lacerda, C.D. Macedo, P. McVeigh, Z. Ning, G. Oliveira, J.P. Overington, J. Parkhill, M. Pertea, R.J. Pierce, A.V. Protasio, M.A. Quail, M.A. Rajandream, J. Rogers, M. Sajid, S.L. Salzberg, M. Stanke, A.R. Tivey, O. White, D.L. Williams, J. Wortman, W. Wu, M. Zamanian, A. Zerlotini, C.M. Fraser-Liggett, B.G. Barrell, N.M. El-Sayed, The genome of the blood fluke *Schistosoma mansoni*, Nature 460 (2009) 352–358.
[43] L.F. Andrade, L.A. Nahum, L.G. Avelar, L.L. Silva, A. Zerlotini, J.C. Ruiz, G. Oliveira, Eukaryotic protein kinases (ePKs) of the helminth parasite *Schistosoma mansoni*, BMC Genomics 12 (2011) 215.
[44] I. Tsai, T.D. Otto, M. Berriman, Improving draft assemblies by iterative mapping and assembly of short reads to eliminate gaps, Genome Biol. 11 (2010) R41.
[45] D.H. Bos, C. Mayfield, D.J. Minchella, Analysis of regulatory protease sequences identified through bioinformatic data mining of the *Schistosoma mansoni* genome, BMC Genomics 10 (2009) 488.
[46] The *Schistosoma japonicum* genome reveals features of host-parasite interplay, Nature 460 (2009) 345–351.
[47] N.D. Young, A.R. Jex, B. Li, S. Liu, L. Yang, Z. Xiong, Y. Li, C. Cantacessi, R.S. Hall, X. Xu, F. Chen, X. Wu, A. Zerlotini, G. Oliveira, A. Hofmann, G. Zhang, X. Fang, Y. Kang, B.E. Campbell, A. Loukas, S. Ranganathan, D. Rollinson, G. Rinaldi, P.J. Brindley, H. Yang, J. Wang, R.B. Gasser, Whole-genome sequence of *Schistosoma haematobium*, Nat. Genet. 44 (2012) 221–225.
[48] S. Verjovski-Almeida, R. DeMarco, E.A. Martins, P.E. Guimaraes, E.P. Ojopi, A.C. Paquola, J.P. Piazza, M.Y. Nishiyama Jr., J.P. Kitajima, R.E. Adamson, P.D. Ashton, M.F. Bonaldo, P.S. Coulson, G.P. Dillon, L.P. Farias, S.P. Gregorio, P.L. Ho, R.A. Leite, L.C. Malaquias, R.C. Marques, P.A. Miyasato, A.L. Nascimento, F.P. Ohlweiler, E.M. Reis, M.A. Ribeiro, R.G. Sa, G.C. Stukart, M.B. Soares, C. Gargioni, T. Kawano, V. Rodrigues, A.M. Madeira, R.A. Wilson, C.F. Menck, J.C. Setubal, L.C. Leite, E. Dias-Neto, Transcriptome analysis of the acoelomate human parasite *Schistosoma mansoni*, Nat. Genet. 35 (2003) 148–157.
[49] F. Liu, J. Lu, W. Hu, S.Y. Wang, S.J. Cui, M. Chi, Q. Yan, X.R. Wang, H.D. Song, X.N. Xu, J.J. Wang, X.L. Zhang, X. Zhang, Z.Q. Wang, C.L. Xue, P.J. Brindley, D.P. McManus, P.Y. Yang, Z. Feng, Z. Chen, Z.G. Han, New perspectives on host-parasite interplay by comparative transcriptomic and proteomic analyses of *Schistosoma japonicum*, PLoS Pathog. 2 (2006) e29.
[50] M.T. Swain, D.M. Larkin, C.R. Caffrey, S.J. Davies, A. Loukas, P.J. Skelly, K.F. Hoffmann, *Schistosoma* comparative genomics: integrating genome structure, parasite biology and anthelmintic discovery, Trends Parasitol. 27 (2011) 555–564.
[51] A.V. Protasio, I.J. Tsai, A. Babbage, S. Nichol, M. Hunt, M.A. Aslett, N. De Silva, G.S. Velarde, T.J. Anderson, R.C. Clark, C. Davidson, G.P. Dillon, N.E. Holroyd, P.T. LoVerde, C. Lloyd, J. McQuillan, G. Oliveira, T.D. Otto, S.J. Parker-Manuel, M.A. Quail, R.A. Wilson, A. Zerlotini, D.W. Dunne, M. Berriman, A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*, PLoS Negl. Trop. Dis. 6 (2012) e1455.
[52] A.V. Protasio, D.W. Dunne, M. Berriman, Comparative study of transcriptome profiles of mechanical- and skin-transformed *Schistosoma mansoni* schistosomula, PLoS Negl. Trop. Dis. 7 (2013) e2091.
[53] S. Liu, P. Cai, X. Piao, N. Hou, X. Zhou, C. Wu, H. Wang, Q. Chen, Expression profile of the *Schistosoma japonicum* degradome reveals differential protease expression patterns and potential anti-schistosomal intervention targets, PLoS Comput. Biol. 10 (2014) e1003856.
[54] E. de la Torre-Escudero, R. Perez-Sanchez, R. Manzano-Roman, A. Oleaga, In vivo intravascular biotinylation of *Schistosoma bovis* adult worms and proteomic analysis of tegumental surface proteins, J. Proteomics 94 (2013) 513–526.
[55] R. deMarco, S. Verjovski-Almeida, Schistosomes—proteomics studies for potential novel vaccines and drug targets, Drug Discov. Today 14 (2009) 472–478.
[56] S. Braschi, R.S. Curwen, P.D. Ashton, S. Verjovski-Almeida, A. Wilson, The tegument surface membranes of the human blood parasite *Schistosoma mansoni*: a proteomic analysis after differential extraction, Proteomics 6 (2006) 1471–1482.
[57] S. Braschi, R.A. Wilson, Proteins exposed at the adult schistosome surface revealed by biotinylation, Mol. Cell Proteomics 5 (2006) 347–356.
[58] J. Sotillo, M. Pearson, L. Becker, J. Mulvenna, A. Loukas, A quantitative proteomic analysis of the tegumental proteins from *Schistosma mansoni* schistosomula reveals novel potential therapeutic targets, Int. J. Parasitol. Drugs Drug Resist. 45 (2015) 505–516.
[59] C.L. Cass, J.R. Johnson, L.L. Califf, T. Xu, H.J. Hernandez, M.J. Stadecker, J.R. Yates 3rd, D.L. Williams, Proteomic analysis of *Schistosoma mansoni* egg secretions, Mol. Biochem. Parasitol. 155 (2007) 84–93.
[60] M. Delcroix, K. Medzihradsky, C.R. Caffrey, R.D. Fetter, J.H. McKerrow, Proteomic analysis of adult *S. mansoni* gut contents, Mol. Biochem. Parasitol. 154 (2007) 95–97.
[61] A.J. O'Donoghue, Y. Jin, G.M. Knudsen, N.C. Perera, D.E. Jenne, J.E. Murphy, C.S. Craik, T.W. Hermiston, Global substrate profiling of proteases in human neutrophil extracellular traps reveals consensus motif predominantly contributed by elastase, PLoS One 8 (2013) e75141.
[62] A.J. O'Donoghue, G.M. Knudsen, C. Beekman, J.A. Perry, A.D. Johnson, J.L. DeRisi, C.S. Craik, R.J. Bennett, Destructin-1 is a collagen-degrading endopeptidase secreted by *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 7478–7483.
[63] A.J. O'Donoghue, A.A. Eroy-Reveles, G.M. Knudsen, J. Ingram, M. Zhou, J.B. Statnekov, A.L. Greninger, D.R. Hostetter, G. Qu, D.A. Maltby, M.O. Anderson, J.L. Derisi, J.H. McKerrow, A.L. Burlingame, C.S. Craik, Global identification of peptidase specificity by multiplex substrate profiling, Nat. Methods 9 (2012) 1095–1100.
[64] J. Dvorak, S.T. Mashiyama, M. Sajid, S. Braschi, M. Delcroix, E.L. Schneider, W.H. McKerrow, M. Bahgat, E. Hansell, P.C. Babbitt, C.S. Craik, J.H. McKerrow, C.R. Caffrey, SmCL3, a gastrodermal cysteine protease of the human blood fluke *Schistosoma mansoni*, PLoS Negl. Trop. Dis. 3 (2009) e449.
[65] D.G. Colley, S.K. Wikel, *Schistosoma mansoni*: simplified method for the production of schistosomules, Exp. Parasitol. 35 (1974) 44–51.
[66] S. Stefanic, J. Dvorak, M. Horn, S. Braschi, D. Sojka, D.S. Ruelas, B. Suzuki, K.C. Lim, S.D. Hopkins, J.H. McKerrow, C.R. Caffrey, RNA interference in *Schistosoma mansoni* schistosomula: selectivity, sensitivity and operation for larger-scale screening, PLoS Negl. Trop. Dis. 4 (2010) e850.
[67] L. Rojo-Arreola, T. Long, D. Asarnow, B.M. Suzuki, R. Singh, C.R. Caffrey, Chemical and genetic validation of the statin drug target to treat the helminth disease, schistosomiasis, PLoS ONE 9 (2014) e87594.
[68] P.F. Basch, Cultivation of *Schistosoma mansoni in vitro*. I. Establishment of cultures from cercariae and development until pairing, J. Parasitol. 67 (1981) 179–185.
[69] J.P. Salter, K.C. Lim, E. Hansell, I. Hsieh, J.H. McKerrow, Schistosome invasion of human skin and degradation of dermal elastin are mediated by a single serine protease, J. Biol. Chem. 275 (2000) 38667–38673.
[70] J.P. Salter, Y. Choe, H. Albrecht, C. Franklin, K.C. Lim, C.S. Craik, J.H. McKerrow, Cercarial elastase is encoded by a functionally conserved gene family across multiple species of schistosomes, J. Biol. Chem. 277 (2002) 24618–24624.
[71] A.J. O'Donoghue, C.S. Mahon, D.H. Goetz, J.M. O'Malley, D.M. Gallagher, M. Zhou, P.G. Murray, C.S. Craik, M.G. Tuohy, Inhibition of a secreted glutamic peptidase prevents growth of the fungus *Talaromyces emersonii*, J. Biol. Chem. 283 (2008) 29186–29195.
[72] T.C. O'Brien, Z.B. Mackey, R.D. Fetter, Y. Choe, A.J. O'Donoghue, M. Zhou, C.S. Craik, C.R. Caffrey, J.H. McKerrow, A parasite cysteine protease is key to host protein degradation and iron acquisition, J. Biol. Chem. 283 (2008) 28934–28943.
[73] J.L. Small, A.J. O'Donoghue, E.C. Boritsch, O.V. Tsodikov, G.M. Knudsen, O. Vandal, C.S. Craik, S. Ehrt, Substrate specificity of MarP, a periplasmic protease required for resistance to acid and oxidative stress in *Mycobacterium tuberculosis*, J. Biol. Chem. 288 (2013) 12489–12499.
[74] S.K. Sreedharan, C. Verma, L.S. Caves, S.M. Brocklehurst, S.E. Gharbia, H.N. Shah, K. Brocklehurst, Demonstration that 1-trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) is one of the most effective low Mr inhibitors of trypsin-catalysed hydrolysis. Characterization by kinetic analysis and by energy minimization and molecular dynamics simulation of the E-64-beta-trypsin complex, Biochem. J. 316 (Pt 3) (1996) 777–786.
[75] I. Corvo, A.J. O'Donoghue, L. Pastro, N. Pi-Denis, A. Eroy-Reveles, L. Roche, J.H. McKerrow, J.P. Dalton, C.S. Craik, C.R. Caffrey, J.F. Tort, Dissecting the active site of the collagenolytic cathepsin L3 protease of the invasive stage of *Fasciola hepatica*, PLoS Negl. Trop. Dis. 7 (2013) e2269.
[76] Y. Choe, F. Leonetti, D.C. Greenbaum, F. Lecaille, M. Bogyo, D. Bromme, J.A. Ellman, C.S. Craik, Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities, J. Biol. Chem. 281 (2006) 12824–12832.
[77] C.M. Stack, C.R. Caffrey, S.M. Donnelly, A. Seshaadri, J. Lowther, J.F. Tort, P.R. Collins, M.W. Robinson, W. Xu, J.H. McKerrow, C.S. Craik, S.R. Geiger, R. Marion, L.S. Brinen, J.P. Dalton, Structural and functional relationships in the virulence-associated cathepsin L proteases of the parasitic liver fluke, *Fasciola hepatica*, J. Biol. Chem. 283 (2008) 9896–9908.
[78] C.R. Caffrey, C.E. Rheinberg, H. Mone, J. Jourdane, Y.L. Li, A. Ruppel, *Schistosoma japonicum*, *S. mansoni*, *S. haematobium*, *S. intercalatum*, and *S. rodhaini*: cysteine-class cathepsin activities in the vomitus of adult worms, Parasitol. Res. 83 (1997) 37–41.
[79] A.J. Barrett, H. Kirschke, B. Cathepsin, H. Cathepsin, L. cathepsin, Methods Enzymol. 80 (Pt C) (1981) 535–561.
[80] M. Delcroix, M. Sajid, C.R. Caffrey, K.C. Lim, J. Dvorak, I. Hsieh, M. Bahgat, C. Dissous, J.H. McKerrow, A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite, J. Biol. Chem. 281 (2006) 39316–39329.
[81] J. Tort, P.J. Brindley, D. Knox, K.H. Wolfe, J.P. Dalton, Proteinases and associated genes of parasitic helminths, Adv. Parasitol. 43 (1999), 161–266.
[82] C.L. Chappell, M.H. Dresden, *Schistosoma mansoni*: proteinase activity of

"hemoglobinase" from the digestive tract of adult worms, Exp. Parasitol. 61 (1986) 160–167.
[83] S.L. Hall, S. Braschi, M. Truscott, W. Mathieson, I.M. Cesari, R.A. Wilson, Insights into blood feeding by schistosomes from a proteomic analysis of worm vomitus, Mol. Biochem. Parasitol. 179 (2011) 18–29.
[84] G. Rinaldi, M.E. Morales, Y.N. Alrefaei, M. Cancela, E. Castillo, J.P. Dalton, J.F. Tort, P.J. Brindley, RNA interference targeting leucine aminopeptidase blocks hatching of *Schistosoma mansoni* eggs, Mol. Biochem. Parasitol. 167 (2009) 118–126.
[85] E. McCarthy, C. Stack, S.M. Donnelly, S. Doyle, V.H. Mann, P.J. Brindley, M. Stewart, T.A. Day, A.G. Maule, J.P. Dalton, Leucine aminopeptidase of the human blood flukes, *Schistosoma mansoni* and *Schistosoma japonicum*, Int. J. Parasitol. 34 (2004) 703–714.
[86] C.K. Sung, M.H. Dresden, Cysteinyl proteinases of *Schistosoma mansoni* eggs: purification and partial characterization, J. Parasitol. 72 (1986) 891–900.
[87] H.L. Asch, M.H. Dresden, Acidic thiol proteinase activity of *Schistosoma mansoni* egg extracts, J. Parasitol. 65 (1979) 543–549.
[88] T.P. Yoshino, M.J. Lodes, A.A. Rege, C.L. Chappell, Proteinase activity in miracidia, transformation excretory-secretory products, and primary sporocysts of *Schistosoma mansoni*, J. Parasitol. 79 (1993) 23–31.
[89] P.D. Ashton, R. Harrop, B. Shah, R.A. Wilson, The schistosome egg: development and secretions, Parasitology 122 (2001) 329–338.
[90] S. Pino-Heiss, M. Brown, J.H. McKerrow, *Schistosoma mansoni*: degradation of host extracellular matrix by eggs and miracidia, Exp. Parasitol. 59 (1985) 217–221.
[91] E. Deu, M. Verdoes, M. Bogyo, New approaches for dissecting protease functions to improve probe development and drug discovery, Nat. Struct. Mol. Biol. 19 (2012) 9–16.
[92] L.E. Sanman, M. Bogyo, Activity-based profiling of proteases, Annu. Rev. Biochem. 83 (2014) 249–273.
[93] J.W. Chang, A.B. Cognetta 3rd, M.J. Niphakis, B.F. Cravatt, Proteome-wide reactivity profiling identifies diverse carbamate chemotypes tuned for serine hydrolase inhibition, ACS Chem. Biol. 8 (2013) 1590–1599.
[94] N. Colaert, K. Helsens, L. Martens, J. Vandekerckhove, K. Gevaert, Improved visualization of protein consensus sequences by iceLogo, Nat. methods 6 (11) (2009) 786–787.

Publikace č. 2

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Trypsin- and Chymotrypsin-Like Serine Proteases in *Schistosoma mansoni* – 'The Undiscovered Country'

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Abstract

Background: Blood flukes (*Schistosoma* spp.) are parasites that can survive for years or decades in the vasculature of permissive mammalian hosts, including humans. Proteolytic enzymes (proteases) are crucial for successful parasitism, including aspects of invasion, maturation and reproduction. Most attention has focused on the 'cercarial elastase' serine proteases that facilitate skin invasion by infective schistosome larvae, and the cysteine and aspartic proteases that worms use to digest the blood meal. Apart from the cercarial elastases, information regarding other *S. mansoni* serine proteases (SmSPs) is limited. To address this, we investigated SmSPs using genomic, transcriptomic, phylogenetic and functional proteomic approaches.

Methodology/Principal Findings: Genes encoding five distinct SmSPs, termed SmSP1 - SmSP5, some of which comprise disparate protein domains, were retrieved from the *S. mansoni* genome database and annotated. Reverse transcription quantitative PCR (RT- qPCR) in various schistosome developmental stages indicated complex expression patterns for SmSPs, including their constituent protein domains. SmSP2 stood apart as being massively expressed in schistosomula and adult stages. Phylogenetic analysis segregated SmSPs into diverse clusters of family S1 proteases. SmSP1 to SmSP4 are trypsin-like proteases, whereas SmSP5 is chymotrypsin-like. In agreement, trypsin-like activities were shown to predominate in eggs, schistosomula and adults using peptidyl fluorogenic substrates. SmSP5 is particularly novel in the phylogenetics of family S1 schistosome proteases, as it is part of a cluster of sequences that fill a gap between the highly divergent cercarial elastases and other family S1 proteases.

Conclusions/Significance: Our series of post-genomics analyses clarifies the complexity of schistosome family S1 serine proteases and highlights their interrelationships, including the cercarial elastases and, not least, the identification of a 'missing-link' protease cluster, represented by SmSP5. A framework is now in place to guide the characterization of individual proteases, their stage-specific expression and their contributions to parasitism, in particular, their possible modulation of host physiology.

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Introduction

Schistosomiasis caused by *Schistosoma* blood flukes is a chronic disease with more than 200 million people infected [1]. Schistosome larvae (cercariae), released into an aquatic environment from snail intermediate hosts, penetrate human skin and subsequently develop into adult worms. Adult worms reside in the host vascular system as male/female pairs, and survive for many years, if not decades [2], producing hundreds of eggs per day. Morbidity arises from the host immune responses to eggs in tissues [3]. Treatment relies on one drug, praziquantel, and no effective vaccine has yet been developed [4]. During its complex life cycle, the parasite survives in various environments by presenting or releasing bioactive molecules that aid survival and modulate host physiology [5,6]. Disruption of these potential mechanisms by specific drugs/vaccines may provide therapeutic benefits.

Proteolysis is a fundamental physiologic process [7,8]. Proteases (proteolytic enzymes) are crucial to parasitism, including by

Author Summary

Schistosomes are blood flukes that live in the blood system and cause chronic and debilitating infection in hundreds of millions of people. Proteolytic enzymes (proteases) produced by the parasite allow it to survive and reproduce. We focused on understanding the repertoire of trypsin- and chymotrypsin-like *Schistosoma mansoni* serine proteases (SmSPs) using a variety of genomic, bioinformatics, RNA- and protein-based techniques. We identified five SmSPs that are produced at different stages of the parasite's development. Based on bioinformatics and cleavage preferences for small peptide substrates, SmSP1 to SmSP4 are trypsin-like, whereas SmSP5 is chymotrypsin-like. Interestingly, SmSP5 forms part of a 'missing link' group of enzymes between the specialized chymotrypsin-like 'cercarial elastases' that help the parasite invade human skin and the more typical chymotrypsins and trypsins found in the nature. Our findings form a basis for further exploration of the functions of the individual enzymes, including their possible contributions to influencing host physiology.

schistosomes, in facilitating invasion, nutrient intake, hatching, excystment, immune evasion [9,10] and modulation of host physiology [10–15]. Most schistosome research has focused either on cysteine and aspartic proteases (MEROPS database Clans CA and AA, respectively [8]), which are responsible for digesting the blood meal [16,17] or on the serine proteases (SPs), known as cercarial elastases (CEs; Clan PA, family S1) that facilitate active penetration of the mammalian host [18–20].

Regarding the nomenclature for eukaryotic SPs, whereas members of the S1 or 'chymotrypsin' family of SPs share a similar tertiary structure, their substrate cleavage specificities differ [8]. Thus, substrate preferences at the P1 subsite [21] may be divided into trypsin-like (P1 preference for basic residues), chymotrypsin-like (bulky hydrophobic residues) and elastase-like (small aliphatic residues) [7].

Despite their name, which was derived from their ability to cleave insoluble elastin, the *S. mansoni* CEs have a chymotrypsin-like P1 specificity [22] due to preferences for phenylalanine and leucine. In contrast to these well-studied CEs [18–20], there are fewer descriptions of 'non-CE' Clan PA, family S1 serine proteases in *S. mansoni* (SmSPs) [6,12–15,23,24].

Among these, SmSP1 (*S. mansoni* serine protease 1, GenBank AJ011561), has been partially described [13,14]. The open reading frame (ORF) of SmSP1 comprises two non-proteolytic domains, followed by a C-terminal trypsin protease domain. Expression of the trypsin domain (mRNA and protein) was noted in adult worms with a significant accumulation in the tegument (surface) of males [13]. Another SmSP was identified (under TC16843 code) by microarray analysis with a remarkably elevated expression in post-infective larvae (schistosomula) that had been maintained *in vitro* [23]. Two additional biochemical studies support a function for schistosome SPs in modulating host physiology. Specifically, a protein fraction of *S. mansoni* adult worm extracts was shown to possess kallikrein-like protease activity [12]. The isolated native enzyme, termed sK1, cleaved kallikrein substrates and processed kininogen to bradykinin which induced strong vasodilatation and decreased arterial blood pressure in experimental rats; sK1 was found in higher abundance in males [12]. Both, sK1 and SmSP1, are proposed to regulate host vascular functions [6]. In the second study, SP activity in extracts of *S. mansoni* eggs induced significant fibrinolytic activity and was associated with a 27 kDa protein [15]. This protease activity had a similar cleavage pattern to human plasmin and it was hypothesized that the enzyme blocks the intravascular deposition of fibrin by platelets activated by schistosome eggs [15].

In the present study, we sought to understand the gene repertoire of non-cercarial elastase SmSPs by employing a series of genomic, transcriptomic, proteolytic and phylogenetic approaches. In addition to SmSP1, we identified and re-annotated four distinct SmSPs in the *S. mansoni* GeneDB genome database [25,26] and term them SmSP2 through SmSP5 according to a previous terminology [13]. The data reveal intriguing expression profiles and phylogenetic relationships that stimulate further study of the individual proteases involved, and their contributions to modulating host physiology.

Materials and Methods

Ethics statement

Mice are kept in the animal facility of the Biology Center (Academy of Sciences of the Czech Republic) in Ceske Budejovice and all animal experiments are carried out as approved by the Animal Rights Ethics Committee under protocol no. 068/2010 issued according to the national regulation 246/1992 Sb.

Schistosome material

A Liberian isolate of *S. mansoni* has been maintained in the laboratory by cycling between CD-1 mice and the freshwater snail, *Biomphalaria glabrata*. Mice were subcutaneously injected with 200 cercariae and sacrificed 6–7 weeks post-infection by intraperitoneal injection of thiopental (50 mg/kg). Adults, eggs and miracidia were isolated as described previously [27]. Cercariae were obtained from infected snails induced to release the parasite under a light stimulus. Cercariae were chilled on ice, collected and transformed mechanically to schistosomula [27,28], which were then cultured for five days under a 5% CO_2 atmosphere at 37°C in Basch Medium 169 [29] containing 5% fetal calf serum and 1% ABAM (antibiotics/antimycotics; Sigma-Aldrich). Daughter sporocyst material was isolated by excision of the hepato-pancreases from two month-infected *B. glabrata* snails. The hepato-pancreases from uninfected snails were used as a negative control when evaluation gene expression.

Isolation of mRNA and cDNA synthesis

Adult worms, eggs, miracidia, daughter sporocysts, cercariae and schistosomula were re-suspended in 500 µl of Trizol reagent (Life Sciences) and processed [30]. Single-stranded cDNA was synthesized from total RNA by SuperScript II reverse transcriptase (Life Sciences) and an oligo dT_{18} primer, and then stored at −20°C.

Gene annotation, domain expression evaluation and sequencing

Genes encoding complete SmSPs or their specific domains were retrieved from the *S. mansoni* genome database (*S. mansoni* GeneDB, available at http://www.genedb.org/Homepage/Smansoni) through BLAST searches. Amino acid sequences of vertebrate family S1 SPs were used as queries. Specific PCR primers were employed to amplify each of the sequences retrieved, and the respective amplicons cloned into the TOPO TA 2.1 vector (Life Technologies) for propagation in TOP10 *E. coli* cells. For SmSP4 and SmSP5, full-length sequences were obtained by 5′ and 3′ RACE (Rapid Amplification of cDNA Ends, Life Technologies).

Based on more recent annotations, the original sequence information for SmSP4 and SmSP5 (GenBank XM_002572739

and XM_002574902) were corrected in the *S. mansoni* GeneDB database. All newly described SmSP sequences were deposited in GenBank under the accession numbers listed in Table 1. For genes with multi-domain structures, PCR analysis was performed using domain-specific primers in order to detect possible differential expression.

Evaluation of gene expressions by RT-qPCR analysis

Gene expression of the SmSPs was assessed using RT-qPCR. For genes with multi-domain structures (SmSP1 and SmSP3), the expression levels of individual domains were evaluated separately. cDNA for various life stages was generated using the mRNA isolation protocol described above and previously [30]. For mRNA isolation, 3 infected *B. glabrata* hepatopancreases and approximately 20 adult pairs, 500 hundred eggs, cercariae and schistosomula were used. Primers for quantitative PCR analysis were designed using the Primer 3 software (http://frodo.wi.mit.edu/ [31],), in order to amplify 150–250 bp regions of the targeted genes or their domains. Primer efficiency was evaluated by serial dilutions of both the primers and the cDNA template as described [32,33]. Two to three primer pairs were generated per target from which one primer set with optimal efficiency and generating only a single dissociation peak was used (see Supporting Information Table S1).

Reactions, containing SYBR Green I Mastermix (Eurogentech), were prepared in final volumes of 25 µL in 96-well plates [30]. The amplification profile consisted of an initial hot start (95°C for 10 min), followed by 40 cycles comprising 95°C for 30 s, 55°C for 60 s and 72°C for 60 s, and ended with a single cycle of 95°C for 60 s, 55°C for 30 s and 95°C for 30 s. PCR reactions were performed in duplicate for each cDNA sample. At least one biological replicate, i.e., samples from a different RNA isolation was performed for each gene target. Analysis of the cycle threshold (C_T) for each target was carried out as described [30] and employed *S. mansoni* cytochrome C oxidase I (SmCOX I, GenBank AF216698, [33]) as the sample normalizing gene transcript [27]. Finally, the resulting transcript values were calculated as a percentage of the expression of the normalizing gene (SmCOX I) which was set as 100%. Transcript levels were expressed as log functions and as a percentage relative to that of SmCOX I in order to compare variable expression patterns. The threshold for significance of expression was set to 0.01% of the expression of SmCOX I.

Phylogenetic analyses of SmSPs

The amino acid sequences of 96 vertebrate and invertebrate members of the S1 serine protease family were aligned in MAFFT [34] using the E-INS-i method, and gap opening (–op) and extension penalties (–ep) of 5.0 and 0.0, respectively. The non-catalytic domains and N-terminal extensions were excluded from the resulting alignment in BioEdit (v7.0.5.2; [35]). The bacterial trypsin from *Streptomyces griseus* was used as an outgroup. The list of family S1 proteases (SPs sequences) used for the phylogenetic analysis is in the Supplementary Table S2. The Maximum Parsimony analysis was performed in PAUP* (v4.b10; [36]), using a heuristic search with random taxa addition, the ACCTRAN option, and the TBR swapping algorithm. All characters were treated as unordered whereas gaps were treated as missing data. Maximum Likelihood analysis was performed in RAxML under the WAG model [37]. Clade support values were calculated from 1000 bootstrap replicates with random sequence additions for both analyses. All trees were displayed using the TreeView32 program [38].

Table 1. List of studied serine proteases and their accession numbers.

Name	SchistoDB	GenBank
SmSP1	Smp_030350	KF535923
SmSP2	Smp_002150	KF510120
SmSP3	Smp_103680	KF510121
SmSP4	Smp_129230	KF510122
SmSP5	Smp_141450	KF939306

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Collection of E/S products and soluble protein extracts

Fifty pairs of adult worms, 1 000 eggs or 1 000 schistosomula were washed five times in Basch Medium 169 containing 1% Fungizone (Gibco) and allowed to stand for 1 h at 37°C in 5% CO_2. Samples were washed 10 times and then incubated in the same Basch Medium overnight (adults and eggs) or for five days (schistosomula) at 37°C in 5% CO_2. Parasite material was then washed 10 times in M-199 medium (alternative medium for schistosoma cultivation without serum and proteins,Gibco) containing 1% ABAM and incubated in the same medium for 16 h at 37°C in 5% CO_2. Medium containing E/S products was removed and filtered using an Ultrafree-MC 0.22 µm filter (Millipore). Filtered medium was buffer exchanged into ice-cold 1× PBS (pH 7.4) and concentrated at 4°C to a 2 ml final volume by centrifugation at 4000 *g* using an Amicon 10000 Ultra-15 Centrifugal Filter Unit (Millipore). The total volume of PBS used for buffer exchange was 40 ml. Samples (0.04–0.37 mg protein/ml) were frozen in liquid nitrogen and stored at −80°C.

Soluble protein extracts (1–5 mg protein/ml) from *S. mansoni* adults, eggs and 5 day-old schistosomula were prepared by homogenization in 50 mM Tris-HCl buffer, pH 8.0, containing 1% CHAPS, 1 mM EDTA and 10 µM of the cysteine protease inhibitor, E-64, in an ice bath. The extracts were cleared by centrifugation (16,000 *g*, 10 min, 4°C), filtered with an Ultrafree-MC 0.22 µm and stored at −80°C.

Proteolytic activity measurement

Proteolytic activities were measured in a kinetic continuous assay using the following peptidyl fluorogenic, 7-amino-4-methylcoumarin (AMC) substrates (Bachem) at a 50 µM final concentration: Z-F-R-AMC (Z, Benzyloxycarbonyl), Bz-F-V-R-AMC (Bz, Benzoyl), Z-G-P-R-AMC, P-F-R-AMC, Boc-L-R-R-AMC (Boc, t-Butyloxycarbonyl), Boc-Q-A-R-AMC, Boc-V-L-K-AMC Suc-A-A-F-AMC (Suc, Succinyl), Suc-A-A-P-F-AMC, Suc-L-Y-AMC, MeOSuc-A-A-P-V-AMC (MeOSuc, 3-Methoxysuccinyl), Z-G-G-L-AMC and Z-V-K-M-AMC. Assays were performed at 37°C in 96-well black microplates in a total volume of 100 µl. Parasite extracts (1–3 µg) or E/S products (0.05–1 µg) were pre-incubated for 10 min in 150 mM Tris-HCl, pH 8.0, containing 10 µM E64, 1 mM EDTA in the presence or absence of 0.5 mM of the serine protease inhibitors, Pefabloc SC and PMSF. E64 was included routinely in extract preparations in order to inhibit Clan CA cysteine protease activity that is present in the life-stages examined [30,39,40]. Hydrolysis of substrate was measured continuously using an Infinite M1000 microplate reader (Tecan) at excitation and emission wavelengths of 360 and 465 nm, respectively. All measurements were performed in triplicate and results normalized to protein concentration.

Molecular modeling

A spatial model of SmSP1 was constructed using the template X-ray structure of bovine trypsin in complex with the peptidyl inhibitor leupeptin (PDB entry 1JRT) and utilizing a pairwise sequence alignment generated by the BLAST program (BLOSUM62 substitution matrix). The homology module of the MOE program was used for modeling the SmSP1 structure (MOE: Chemical Computing Group; http://www.chemcomp.com). The conformation of leupeptin was refined by applying the LigX module of the MOE. The final binding mode of the inhibitor was selected by the best fit model based on the London dG scoring function and the generalized Born method [41]. Molecular images were generated with UCSF Chimera (http://www.cgl.ucsf.edu/chimera/). The electrostatic surface potential was calculated using the APBS software [42] and input data were prepared using PDB2PQR [43].

Results

Gene annotation and sequence analysis reveals complex domain organizations for some SmSPs

Genes were selected *in silico* based on a proteolytic domain organization that matched with family S1 serine proteases: cercarial elastases were excluded because of their detailed studies previously [20,22]. The five remaining SmSP genes, including the previously sequenced and partially characterized SmSP1 [13,14], were cloned and sequenced. The other four gene sequences named SmSP2 through SmSP5 (Table 1) were significantly corrected and re-annotated in the primary database (*S. mansoni* GeneDB) due to various sequence inaccuracies. The sequences of SmSP2 through SmSP5 were deposited into the GenBank as KF510120, KF510121, KF510122, KF939306, respectively. The sequence of SmSP1 defined here was also deposited (KF535923) because of sequence differences from the original description (CAA09691 [13]) and from the information in *S. mansoni* GeneDB (Smp_030350; Figure S1). A search of the *Schistosoma japonicum* genome [44] indicates that orthologs for each of the SmSPs are present; SjSP1 (GeneDB Sjp_0012180, GenBank N/A), SjSP2 (Sjp_0100980, CAX74751), SjSP3 (Sjp_0023390, CAX73257), SjSP4 (Sjp_0047680, N/A) and SjSP5 (Sjp_0114710, CAX73292).

The sequence domain organization for the particular proteases is represented in Figure 1. Based on sequence homology analysis, we describe SmSP1 as a multi-domain protein comprising a matriptase-like structure made up of **C**omplement-**U**egf-**B**MP-1 (CUB) extracellular and plasma membrane-associated domains, a LDL-binding receptor domain class A (LDLa domain) and a S1 family serine protease domain. However, the full gene product has been detected only in the eggs, whereas in other parasite stages, the CUB and protease domains are expressed as separate spliced products, as demonstrated by PCR and sequencing (Figure S2).

Primary sequence homology analysis shows that SmSP2 to SmSP5 are distinct molecules with the same family S1 type catalytic protease domain at the C-terminus, but with different N-terminal extensions which include a potential pro-peptide, i.e., a peptide that is removed during zymogen activation. The N-terminal extensions vary from 201 residues in SmSP2 to just a seven residues in SmSP5 (Figure 1). SmSP1, SmSP3 and SmSP5 do not contain a predicted signal sequence for the secretory pathway as identified by the SignalP program [45]. In contrast, SmSP2 and SmSP4 are synthesized as pre-pro-proteins with a typical N-terminal signal peptide preceding an N-terminal extension region containing a putative pro-peptide ('activation peptide') that is then followed by the protease domain (Figure 1). The pro-peptide is separated from the protease domain of SmSPs by a basic residue, Arg or Lys (Figure 2) which constitutes a potential activating cleavage site, i.e., is hydrolyzed during protease maturation as is known for other S1 family proteases [7]. For SmSP3, the N-terminal extension contains an incomplete CUB domain. PCR and sequencing revealed that, as found for SmSP1, the CUB and the protease domains of SmSP3 are only co-expressed in eggs whereas they are separate spliced gene products in the other stages (Figure S2). SmSP5 contains a Thr/Asn rich C-terminal sequence extension not present in orthologous SPs from other trematodes (Figure S4).

The catalytic protease domains of SmSP1 to SmSP4 share significantly greater sequence identity (about 30%) with each other than with SmSP5 (about 20%; Figure S3). All five SmSPs have a catalytic triad in the order of His, Asp and Ser that is typical for S1 family proteases; also, the regions surrounding the catalytic triad residues have the most notable sequence identity (Figure 2). The protease domains of SmSP1 to SmSP4 contain cysteine residues at positions 28, 44, 130, 160, 173, 184, 194, and 212 (SmSP1 protease domain numbering), which are conserved in other trypsin-like proteases. They form four disulfide bonds that can be predicted from the alignment with the crystal structures of bovine trypsin and bovine chymotrypsin (Figure 2). Moreover, the protease domain of SmSP2 through SmSP4 contains an additional cysteine residue, Cys112. By comparison with bovine chymotrypsin, this residue in SmSP2 and SmSP3 is likely to form a disulfide bond with a Cys in the N-terminal extension region (at the positions -p13 and -p9, respectively), whereas in SmSP4 a similar Cys in the N-terminal extension region is lacking (Figure 2).

SmSP5 diverges from the other four SPs in that it contains only six cysteine residues that likely form three disulfide bonds. The first two bonds, Cys28-Cys44 and Cys160-Cys173, are identical to those in trypsin, chymotrypsin and other SmSPs. The remaining cysteine residues (Cys46 and Cys72) are absent, but correspond to Cys46 and Cys77 in SmCE that were predicted to form a disulfide bond by homology modeling [46] (Figure 2). Moreover, both SmSP5 and SmCEs lack the disulfides Cys130-Cys194 and Cys184-Cys212, which are conserved in SmSP1 to SmSP4. Taken together, SmSP5 clearly differs in its disulfide pattern from the other investigated SmSPs. This close structural relationship between SmSP5 and the SmCEs is confirmed for the other analyses performed (see below). In addition, two other splice variants of SmSP5 were detected. Compared to the full-length SmSP5, both are C-terminally truncated and one is missing the crucial His residue from the catalytic triad (Figure S4).

Asp182 determines the trypsin-like specificity of serine proteases for substrates with Arg/Lys in the P1 position [47], and this residue is conserved in all of the SmSPs except SmSP5 (Figure 2), which has Gly. Therefore, it might be the case that SmSP5 displays a substrate specificity similar to that of chymotrypsin/elastase-type proteases which also contain a hydrophobic/uncharged residue in the position 182. The calcium binding site in mammalian trypsins is formed mainly by Glu70 and Glu80 (trypsin numbering, corresponding to Glu60 and Glu70 in SmSP1) [48]. This motif is not strictly conserved in the analyzed SmSP sequences; however, it might be present in a modified functional form in SmSP2, SmSP3 and SmSP4 that contain acidic residues in the close proximity of those locations (Figure 2).

SmSPs, including their domains, are differentially expressed across developmental stages

Messenger RNA transcript levels for the five SmSPs were evaluated in eggs, miracidia, daughter sporocysts, cercariae, schistosomula and adults using RT- qPCR (Figure 3). For SmSP1

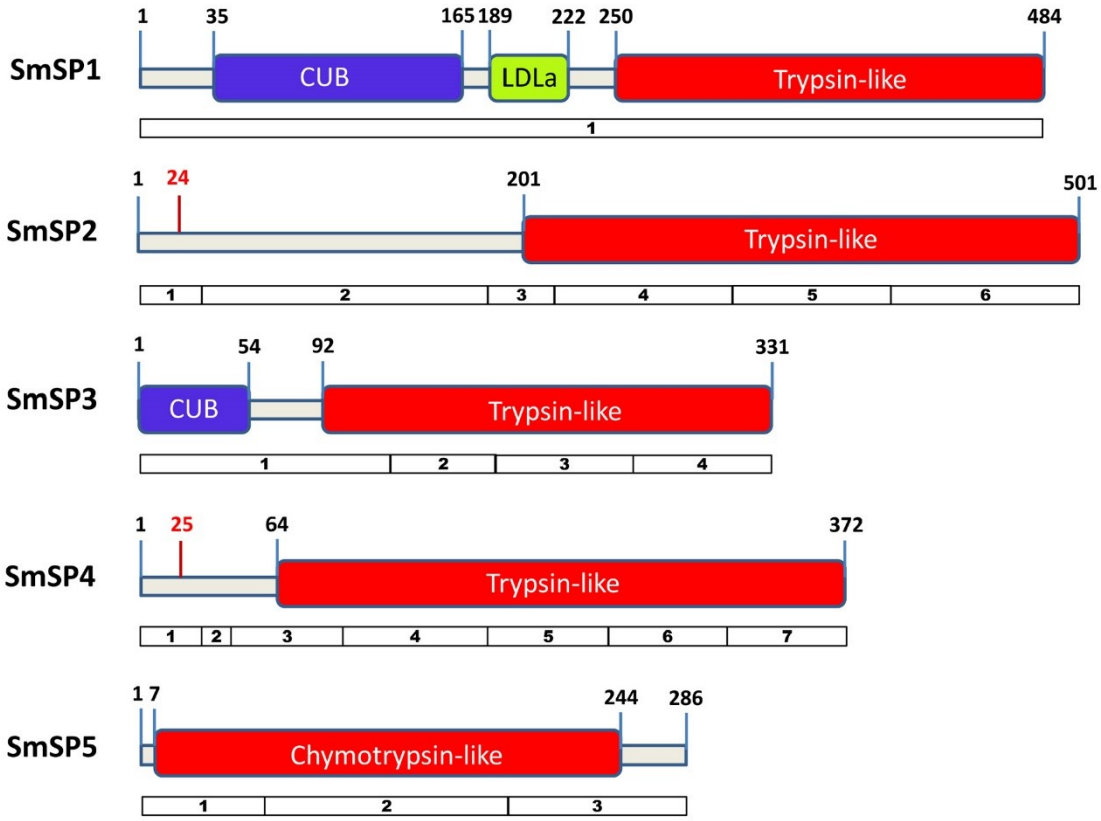

Figure 1. Predicted domain organization and open reading frames of SmSP proteases. CUB domains are depicted in blue, an LDLa domain in yellow and protease domains from the S1 family in red. In SmSP2 and SmSP4, N-terminal signal peptides are separated by red bars from the rest of N-terminal extensions with putative pro-peptides (protease activation peptides). Numbering indicates amino acid positions. Exon structure of the genes encoding SmSPs are shown as numbered boxes below each SmSP protein.
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and SmSP3, we determined gene expression for both the protease and non-protease domains (Figure 4).

For SmSP1, the greatest expression was recorded in eggs at 2.5% of the expression level of the reference gene, SmCOX I. Low expression was recorded in adult worms, five-day old schistosomula and daughter sporocysts at around 0.1% or below relative to SmCOX I. Expression in the other stages was below significance, i.e., less than 0.01% of SmCOX I. As described above, the ORF of SmSP1 consists of 3 domains and their individual expression was evaluated by RT-qPCR and PCR (Figure 4A; Figure S2). The data show a differential expression pattern for the CUB, LDLa and protease domains of SmSP1: expression of the CUB domain is mostly in eggs and sporocysts, whereas LDLa is only expressed in eggs with an expression level about 20-fold lower than that of the protease domain (Figure 4A). As stated above, only in eggs is the whole ORF amplified by PCR suggesting that some SmSP1 is expressed as the full-length multi-domain protein (Figure S2).

Among the SmSPs, SmSP2 is the most abundantly expressed SmSP (Figure 3). In fact, expression in schistosomula and adults is on a similar level to that previously measured for the well-characterized *S. mansoni* cysteine and aspartic proteases [27]. In adults, SmSP2 expression is equivalent to that of SmCOX I, whereas in five-day old schistosomula expression is even greater - 150% that of SmCOX I. Significant expression, i.e., 10% that of SmCOX I, is also detected in eggs. In the other stages, expression is close to or below 1% of the SmCOX I level.

The expression pattern of SmSP3 across all life stages is similar to that of SmSP1 (Figure 3), with minor variations regarding expression in cercariae and schistosomula. Most expression is found in eggs at 2.5% of the SmCOX I expression level. Interestingly, the CUB and protease domains are only co-expressed in eggs and adults (Figure 4B), whereas differential expression is seen for the other developmental stages (Figure S2). SmSP4 is expressed predominantly in eggs (around 10% of SmCOX I level). For the other stages, approximately 1–2% of the SmCOX I level is detectable in cercariae, adults and five-day old schistosomula. Finally, SmSP5 is expressed predominantly in the eggs (2% of the level of SmCOX I) with low expression in the other life stages (0.02–0.05% of SmCOX).

Phylogenetic position of SmSPs: SmSP5 as 'a missing-link' chymotrypsin-like protease

The maximum likelihood analysis of a wide spectrum of vertebrate and invertebrate S1 family SPs based on amino acid sequences revealed that SmSPs clustered with related trematode proteases into five distinct and well-supported clades (Figure 5). Identical results were obtained using maximum parsimony analysis (data not shown). The clades did not create a monophyletic group.

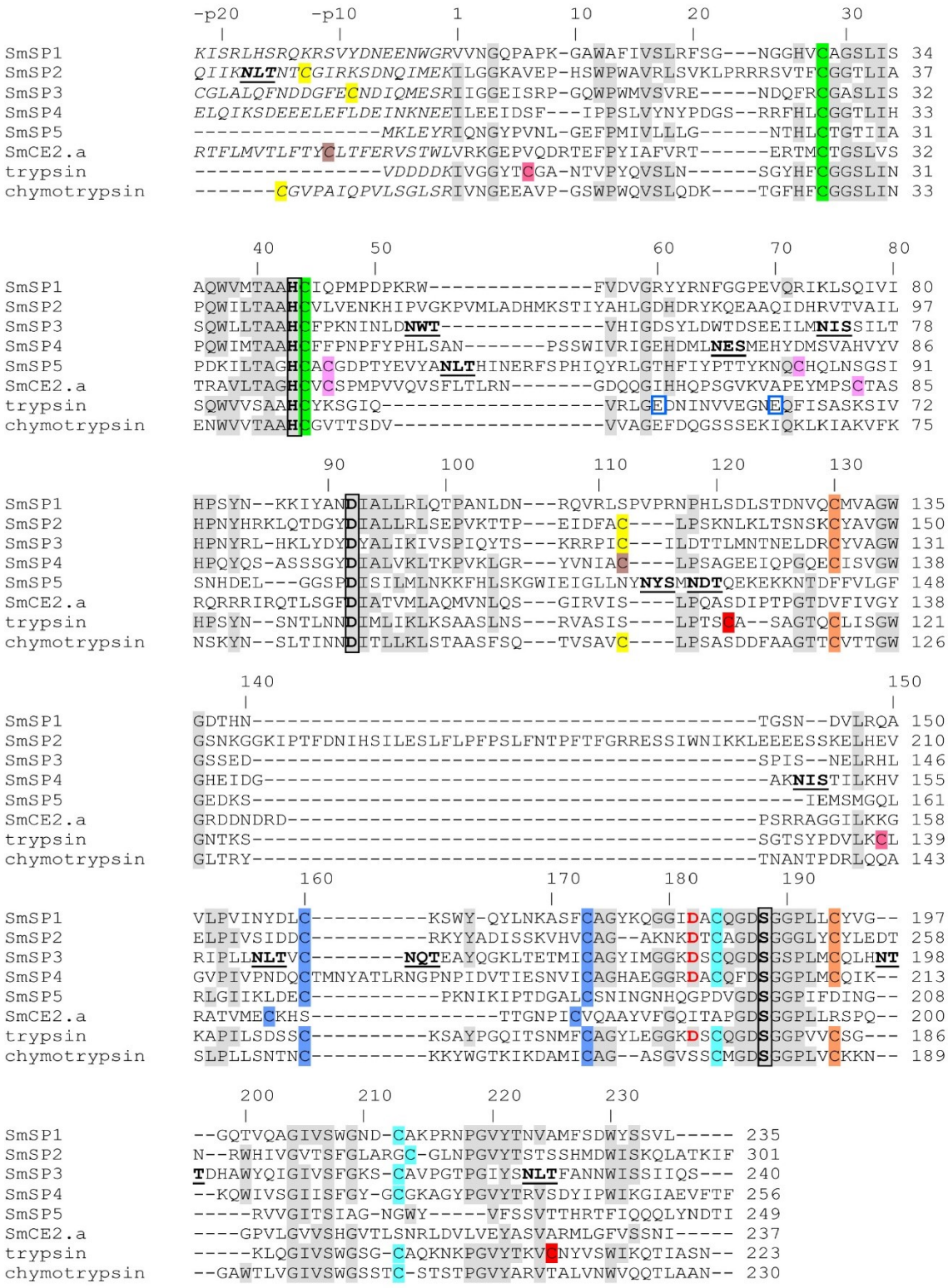
-p20 -p10 1 10 20 30
SmSP1 KISRLHSRQKRSVYDNEENWGRVVNGQPAPK-GAWAFIVSLRFSG---NGGHVCAGSLIS 34
SmSP2 QIIKNLTNTCGIRKSDNQIMEKILGGKAVEP-HSWPWAVRLSVKLPRRRSVTFCGGTLIA 37
SmSP3 CGLALQFNDDGFECNDIQMESRIIGGEISRP-GQWPWMVSVRE-----NDQFRCGASLIS 32
SmSP4 ELQIKSDEEELEFLDEINKNEEILEEIDSF---IPPSLVYNYPDGS--RRFHLCGGTLIH 33
SmSP5 ----------------MKLEYRIQNGYPVNL-GEFPMIVLLLG------NTHLCTGTIIA 31
SmCE2.a RTFLMVTLFTYCLTFERVSTWLVRKGEPVQDRTEFPYIAFVRT------ERTMCTGSLVS 32
trypsin ----------------VDDDDKIVGGYTCGA-NTVPYQVSLN------SGYHFCGGSLIN 31
chymotrypsin -------CGVPAIQPVLSGLSRIVNGEEAVP-GSWPWQVSLQDK----TGFHFCGGSLIN 33
40 50 60 70 80
SmSP1 AQWVMTAAHCIQPMPDPKRW--------------FVDVGRYYRNFGGPEVQRIKLSQIVI 80
SmSP2 PQWILTAAHCVLVENKHIPVGKPVMLADHMKSTIYAHLGDHDRYKQEAAQIDHRVTVAIL 97
SmSP3 SQWLLTAAHCFPKNINLDNWT--------------VHIGDSYLDWTDSEEILMNISSILT 78
SmSP4 PQWIMTAAHCFFPNPFYPHLSAN-------PSSWIVRIGEHDMLNESMEHYDMSVAHVYV 86
SmSP5 PDKILTAGHCACGDPTYEVYANLTHINERFSPHIQYRLGTHFIYPTTYKNQCHQLNSGSI 91
SmCE2.a TRAVLTAGHCVCSPMPVVQVSFLTLRN-------GDQQGIHHQPSGVKVAPEYMPSCTAS 85
trypsin SQWVVSAAHCYKSGIQ--------------------VRLGEDNINVVEGNEQFISASKSIV 72
chymotrypsin ENWVVTAAHCGVTTSDV--------------------VVAGEFDQGSSSEKIQKLKIAKVFK 75
90 100 110 120 130
SmSP1 HPSYN--KKIYANDIALLRLQTPANLDN---RQVRLSPVPRNPHLSDLSTDNVQCMVAGW 135
SmSP2 HPNYHRKLQTDGYDIALLRLSEPVKTTP---EIDFAC----LPSKNLKLTSNSKCYAVGW 150
SmSP3 HPNYRL-HKLYDYDYALIKIVSPIQYTS---KRRPIC---ILDTTLMNTNELDRCYVAGW 131
SmSP4 HPQYQS-ASSSGYDIALVKLTKPVKLGR---YVNIAC----LPSAGEEIQPGQECISVGW 138
SmSP5 SNHDEL---GGSPDISILMLNKKFHLSKGWIEIGLLNYNYSMNDTQEKEKKNTDFFVLGF 148
SmCE2.a RQRRRIRQTLSGFDIATVMLAQMVNLQS---GIRVIS----LPQASDIPTPGTDVFIVGY 138
trypsin HPSYN--SNTLNNDIMLIKLKSAASLNS---RVASIS----LPTSCA--SAGTQCLISGW 121
chymotrypsin NSKYN--SLTINNDITLLKLSTAASFSQ---TVSAVC----LPSASDDFAAGTTCVTTGW 126
140 150
SmSP1 GDTHN----------TGSN--DVLRQA 150
SmSP2 GSNKGGKIPTFDNIHSILESLFLPFPSLFNTPFTFGRRESSIWNIKKLEEEESSKELHEV 210
SmSP3 GSSED----------SPIS--NELRHL 146
SmSP4 GHEIDG----------AKNISTILKHV 155
SmSP5 GEDKS----------IEMSMGQL 161
SmCE2.a GRDDNDRD----------PSRRAGGILKKG 158
trypsin GNTKS----------SGTSYPDVLKCL 139
chymotrypsin GLTRY----------TNANTPDRLQQA 143
160 170 180 190
SmSP1 VLPVINYDLC----------KSWY-QYLNKASFCAGYKQGGIDACQGDSGGPLLCYVG-- 197
SmSP2 ELPIVSIDDC----------RKYYADISSKVHVCAG--AKNKDTCAGDSGGGLYCYLEDT 258
SmSP3 RIPLLNLTVC--------NQTEAYQGKLTETMICAGYIMGGKDSCQGDSGSPLMCQLHNT 198
SmSP4 GVPIVPNDQCTMNYATLRNGPNPIDVTIESNVICAGHAEGGRDACQFDSGGPLMCQIK-- 213
SmSP5 RLGIIKLDEC----------PKNIKIPTDGALCSNINGNHQGPDVGDSGGPIFDING-- 208
SmCE2.a RATVMECKHS----------TTGNPICVQAAYVFGQITAPGDSGGPLLRSPQ-- 200
trypsin KAPILSDSSC----------KSAYPGQITSNMFCAGYLEGGKDSCQGDSGGPVVCSG--- 186
chymotrypsin SLPLLSNTNC----------KKYWGTKIKDAMICAG--ASGVSSCMGDSGGPLVCKKN-- 189
200 210 220 230
SmSP1 --GQTVQAGIVSWGND-CAKPRNPGVYTNVAMFSDWYSSVL----- 235
SmSP2 N--RWHIVGVTSFGLARGC-GLNPGVYTSTSSHMDWISKQLATKIF 301
SmSP3 TDHAWYQIGIVSFGKS-CAVPGTPGIYSNLTFANNWISSIIQS--- 240
SmSP4 --KQWIVSGIISFGY-GCGKAGYPGVYTRVSDYIPWIKGIAEVFTF 256
SmSP5 -----RVVGITSIAG-NGWY-----VFSSVTTHRTFIQQQLYNDTI 249
SmCE2.a ----GPVLGVVSHGVTLSNRLDVLVEYASVARMLGFVSSNI----- 237
trypsin -----KLQGIVSWGSG-CAQKNKPGVYTKVCNYVSWIKQTIASN-- 223
chymotrypsin --GAWTLVGIVSWGSSTC-STSTPGVYARVTALVNWVQQTLAAN-- 230

Figure 2. Primary sequence alignment of SmSP1 through SmSP5 with *S. mansoni* cercarial elastase 2a (SmCE2.a), bovine trypsin and bovine chymotrypsin. For SmSP1 to SmSP4, only the protease domains are shown; the upstream sequences (except a short sequence stretch) forming N-terminal extensions and non-proteolytic domains are not included in the alignment. Also, a downstream C-terminal extension of SmSP5 is not included. The catalytic residues His, Asp and Ser are highlighted in bold and black-boxed; critical Asp residues in the S1 subsite that account for trypsin-like activity are in bold red; Cys residues that are predicted to form disulfide bonds are indicated by the same color; putative unpaired Cys residues are highlighted in olive, and predicted N-glycosylation signals are in bold and underlined. Glu residues binding a Ca^{2+} ion in the trypsin molecule are blue-boxed. The upper line numbering is according to SmSP1; the predicted mature protease domain starts with 1, the suffix p indicates pro-peptide/N-terminal extension numbering. GenBank accession numbers are as follows: SmSP1 (KF535923), SmSP2 (KF510120), SmSP3 (KF510121), SmSP4 (KF510122), SmSP5 (KF939306), SmCE2a (AAM43941), bovine trypsinogen (XP_871686) and bovine chymotrypsinogen A (XP_003583409).
doi:10.1371/journal.pntd.0002766.g002

Thus, SmSP1 and SmSP3 were placed as two closely related but independent clades (trematode SP clade 1 and 3) and clustered with a large group of vertebrate SPs, including regulatory- and epithelial-derived effector trypsin-like proteases such as plasminogens, plasma kallikreins, tryptases, matriptases and transmembrane SPs (Figure 5). SmSP2 and SmSP4 also segregated into two separate but related trematode clades (numbers 2 and 4), which clustered with cestode SPs and a group of insect plasminogen-like and trans-membrane SPs (Figure 5). Finally, SmSP5 clustered with *S. japonicum* and *Clonorchis sinensis* (Chinese liver fluke) orthologs and created a sub-clade that grouped with a sub-clade of CEs within the trematode SP clade 5. This clade also clustered with chymotrypsin-like proteases from invertebrates. Accordingly, SmSP5 and its trematode orthologs associate more with the divergent schistosome CEs [22] than with other S1 family proteases [18].

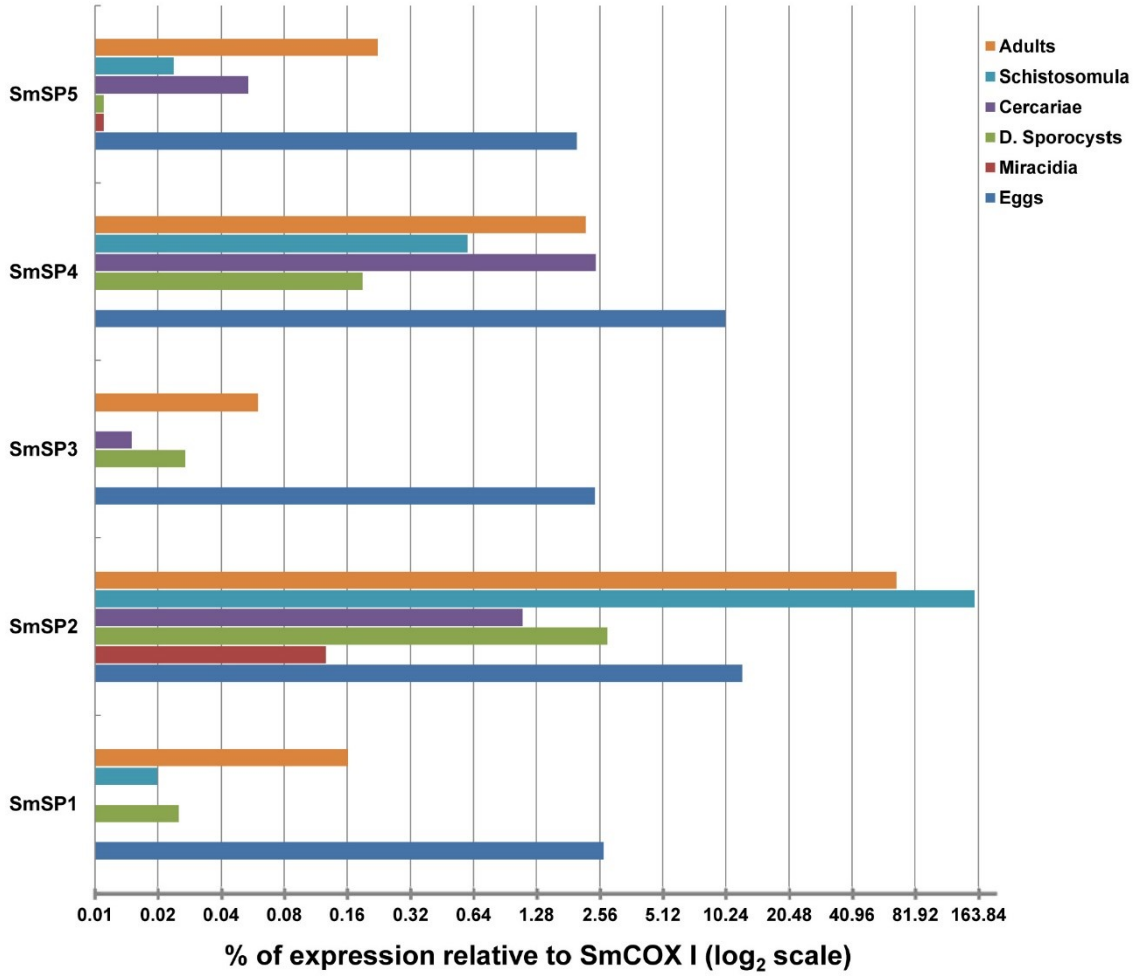

Figure 3. RT-qPCR to evaluate the expression of SmSP genes among *S. mansoni* developmental stages. mRNA levels are displayed as the percentage of expression compared to the constitutively expressed *S. mansoni* cytochrome oxidase I (SmCOX I). The value 0.01% was used as a significance threshold. The gene expression analysis of the protease domains of SmSPs. Each unit represents the -fold change in the transcription level using the $\log_2$ scale.
doi:10.1371/journal.pntd.0002766.g003

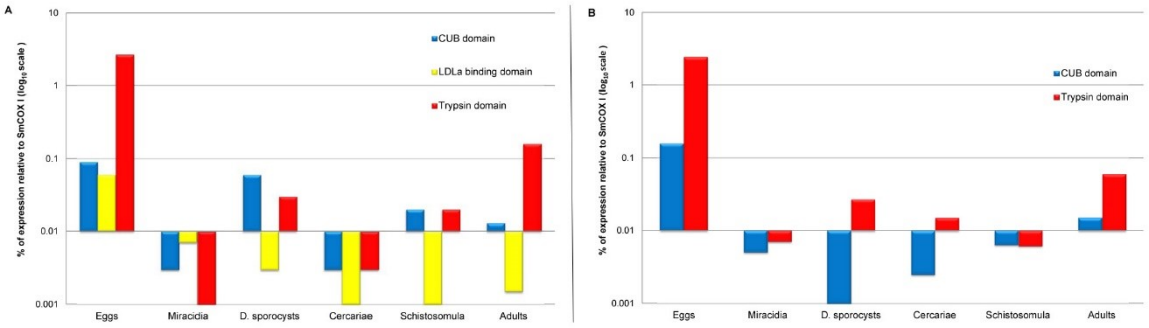

Figure 4. Comparisons of mRNA levels for the separate domains of SmSP1 (A) and SmSP3 (B) displayed in the log_{10} scale and as a percentage of SmCOX I expression level. D. sporocyst = daughter sporocyst.
doi:10.1371/journal.pntd.0002766.g004

Activity profiling demonstrates trypsin-like proteases in *S. mansoni* developmental stages

S1 family SP activities in soluble extracts of *S. mansoni* adults, five day-old schistosomula and eggs were profiled for proteolytic specificity using peptidyl fluorogenic substrates. Two sets of specific protease substrates were used; (i) substrates with a basic amino acid residue (Arg, Lys) in the P1 position that are cleaved by trypsin-like SPs, and (ii) substrates containing bulky hydrophobic (Phe, Tyr) or aliphatic residues (Val, Leu, Met) at P1 that are cleaved by chymotrypsin- or elastase-like SPs [49]. The measured activities were further authenticated as S1 family SPs by their sensitivity to the small molecule inhibitors, Pefabloc SC and PMSF.

The results indicate that trypsin-like activities predominate over chymotrypsin/elastase-like activities in the analyzed extracts (Figure 6). The trypsin substrates were hydrolyzed with variable efficiencies giving distinct cleavage patterns for the individual life stages. The prominent activity in all extracts was best measured with the Boc-L-R-R-AMC substrate, hence making this substrate a useful probe to detect and measure SmSPs. Extracts of eggs displayed a particularly complex profile by cleaving an additional two substrates, Bz-F-V-R-AMC, and Z-G-P-R-AMC. This suggests that this life-stage possesses additional, possibly stage-specific, trypsin-like proteases. In contrast to the major trypsin-like activities, chymotrypsin/elastase-like activity was relatively weak being measured only in schistosomula and adults.

Subsequently, we tested whether SmSPs is measurable in the E/S products from eggs, schistosomula and adults. For this purpose, we used the substrate Boc-L-R-R-AMC, which was identified as the most efficient substrate for homogenates of all the life stages (Figure 6). The specific activities of the E/S products, which were inhibited by the SP inhibitors, Pefabloc SC and PMSF, were 1.05 ± 0.10, 1.38 ± 0.05, and 0.11 ± 0.01 RFU/μg protein for eggs, schistosomula and adults, respectively.

Spatial structure modeling predicts a trypsin-like substrate specificity of SmSP1

A spatial homology model of the protease domain of SmSP1 was constructed to analyze its binding pocket and substrate specificity. The X-ray structure of bovine trypsin in complex with the small-molecule inhibitor, leupeptin (PDB code 1jrt), was used as a template. We used SmSP1 as representative of SmSP1 to SmSP4, which have substantial sequence identity, a similar disulfide pattern and homology in active site regions (Figures 2 and S3). Figure S5 shows that the SmSP1 protease domain displays the conserved architecture of S1 family proteases which consists of two six-stranded β-barrel domains packed against each other. The catalytic amino acid residues are located at the junction between the domains. The major insertion/deletion variations between SmSP1 to SmSP4 (such as the SmSP2 insertion at residue 140, Figure 2) are located at surface-exposed loops.

The primary substrate specificity determinant of S1 family proteases is the S1 binding subsite. In SmSP1, this subsite forms a deep and narrow negatively charged pocket that contains Asp182 at the bottom (Figures 7A and 7B). Leupeptin, the transition state analog protease inhibitor, was docked into the active site of SmSP1. The arginal residue of leupeptin forms a covalent linkage with the catalytic Ser188, a salt bridge with Asp182 in the S1 subsite and hydrogen bonds with the carbonyl oxygen of Ala183 and Asp211 (Figure 7C). An additional hydrogen bond is formed between the side chain nitrogen of Gln185 and the carbonyl oxygen Leu2 residue of leupeptin. The putative interaction pattern of leupeptin at the S1 subsite of SmSP1 is similar to that found in bovine trypsin [50]. This demonstrates that SmSP1 has a substrate binding preference for basic residues at the P1 position, the positive charge of which compliments the negatively charged Asp182, i.e., trypsin-like activity. This conclusion can be generalized to SmSP2 to SmSP4 which also contain the critical Asp182 residue.

Discussion

Much has been reported on the genetic, biochemical and functional characterization of cysteine and aspartic protease activities in schistosomes [16,17] and flatworms in general [16,51], and of the schistosome CE SPs [20] that putatively facilitate parasite invasion of the mammalian host [18–20]. By comparison, relatively little detail is available for non-CE SPs. There are, however, indications that non-CE S1 family SPs contribute to successful infection [6]. Thus, kallikrein-like protease activity from *S. mansoni* adults [12] and plasmin-like fibrinolytic activity from *S. mansoni* eggs [15] have been recorded previously. Both activities displayed trypsin type cleavage specificities and both may contribute to the phenomenon, whereby large occlusions of veins by schistosomes are not associated with intravascular deposition of fibrin and thrombus formation [52–54]. At the gene and primary sequence levels, however, only two SmSPs, namely SmSP1 [13,14] and another [23,24], which we term SmSP2, have been described.

The *S. mansoni* GeneDB currently contains 16 unique sequences that belong to Clan PA family S1 SPs. This number is significantly

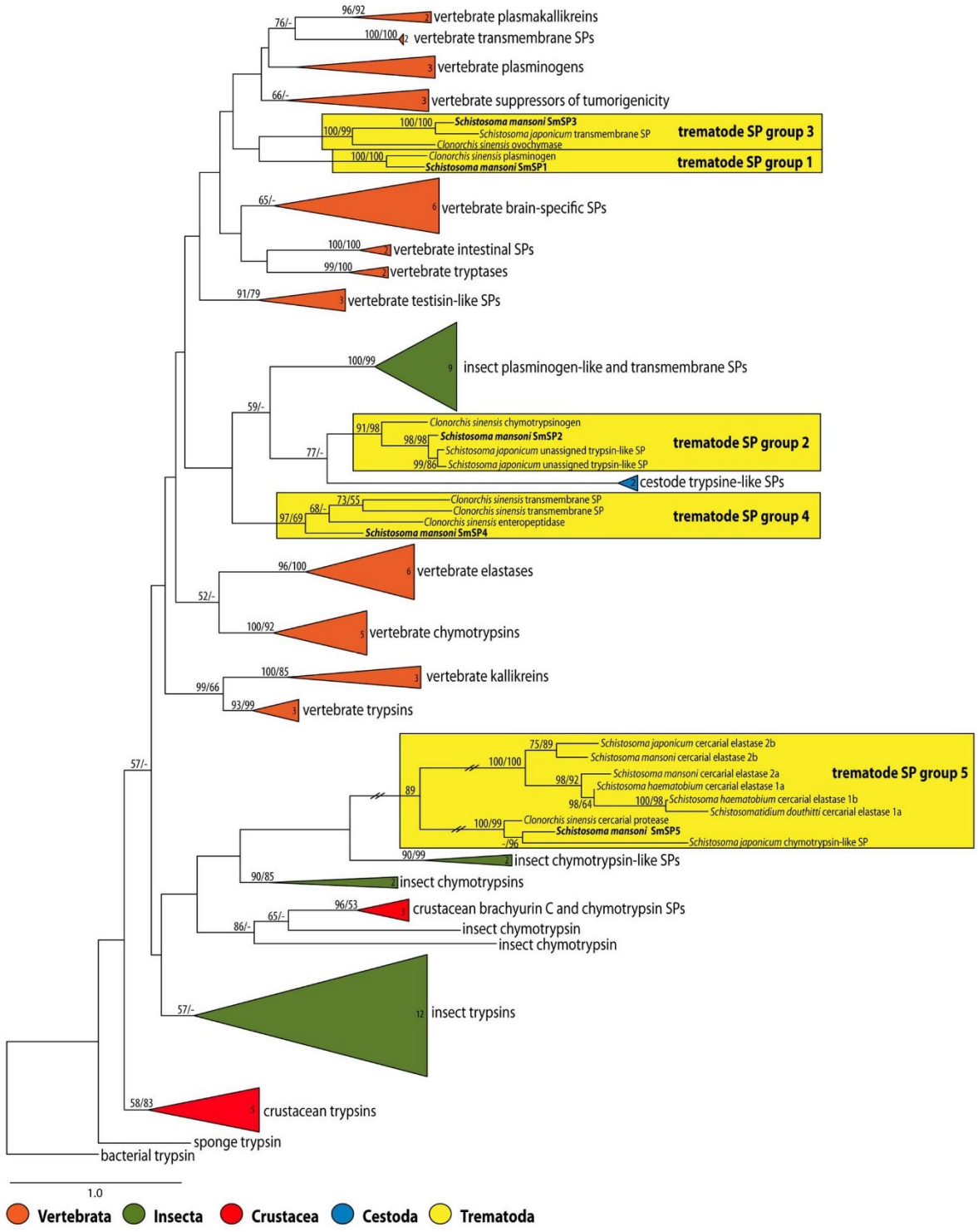

Figure 5. Maximum likelihood phylogenetic tree of 101 selected members of the S1 family of serine proteases with emphasis on trematode SPs. Numbers in the collapsed branches (triangles) indicate the number of taxa included in the branch. Maximum likelihood and maximum parsimony bootstrap supports are shown at nodes, bootstrap percentages with <50% support are not shown. Branches in the trematode clade 5 SPs are shortened to one third of their original length as indicated by the two diagonal lines. For clades 1 and 4, two *S. japonicum* orthologs

are missing due to their absence in the GenBank nr database. However, both sequences can be retrieved from the SchistoDB database under the identifiers Sjp_0012180 (SjSP1) and Sjp_0047680 (Sj SP4).
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lower than the 135 family S1 proteases found in the human genome [8,25] and may be due to the lack of need to regulate the more complex and expanded physiological processes found in vertebrates [55]. In our study and apart from SmSP1 [13,14], we identified four additional SmSP genes encoding typical sequence features of the S1 family [7,8] and which we term SmSP2 through SmSP5. Two further genes (Smp_194090 and Smp_06530 in GeneDB) were identified in the *S. mansoni* GeneDB as putative proteolytically inactive SmSPs as they lack the catalytic serine or histidine residue in the catalytic triad. The remaining nine of the 16 family S1 SPs comprise eight CEs (encoding both putative proteolytically active and inactive products) and a gene (Smp_174530) that encodes an S1 family SP ORF fused downstream of an M01 family metallo-protease. This protease that was not known to us at the beginning of our study and because of its domain complexity and sequence size was not described further.

Our phylogenetic analyses of trematode SPs displayed interesting evolutionary trends. The SmSPs segregate into five clusters of family S1 proteases. The protease domains of SmSP1 and SmSP3, forming clades 1 and 3, respectively, cluster with a large group of vertebrate trypsin-like SPs including regulatory and effector epithelial-derived proteases. In addition to a protease domain, the ORFs for SmSP1 and SmSP3 include non-catalytic CUB domains and SmSP1 LDLa domain. Several vertebrate matriptases that also contain CUB domains are present in our phylogenetic analysis including those belonging to the 'suppressor of tumorigenicity' group. As judged by the domain organization, SmSP1 resembles mammalian matriptases (a.k.a. epithin, MT-SP); however unlike conventional matriptases with multiple CUB and LDLa domains, SmSP1 has only one of each. CUB domains were first described in the complement proteins C1r and C1s and are modules of approximately 110 amino acids with four conserved cysteine residues [56]. These domains mediate protein-protein interactions and are generally associated with proteins that have diverse, usually regulatory, functions in the extracellular space and/or plasma membrane [56]. CUB domains can also interact with heparin and glycoproteins [56] and are often associated with metallo-proteases, in addition to serine proteases [8].

Based on the RT-qPCR analysis, the complete ORFs of SmSP1 and SmSP3 molecules share a similar expression profile (quantitatively and, to a smaller degree, qualitatively) across the developmental stages tested. However, it is also clear that the individual protease, CUB and/or LDLa domains are differentially expressed across the developmental stages tested being only co-expressed in eggs and, for SmSP3, adults. The particular functions of these enzymes and their component domains are unknown and their importance to parasite vitality and/or survival might be tested via specific RNA interference (RNAi), which has been shown to operate in schistosomes [30,57,58]. According to our phylogenetic analysis, the closest vertebrate orthologs to SmSP1 and SmSP3 are those associated with regulatory cascades such as fibrinolysis and vasodilation. This, together with the fact that SmSP1 was detected apparently on the surface area of worms and secreted into the cultivation media [13], suggests a possible function at the host-parasite interface.

The presence in the ORF of SmSP1 of an LDLa domain (positioned between the CUB and catalytic domains) deserves a note. Schistosomes and other flatworms do not synthesize cholesterol (found within LDL) and must therefore scavenge it from the environment, particularly for the energy-intensive work of producing eggs [59,60]. There is also a report that the presence of *S. mansoni* eggs is connected with decreased circulating levels of cholesterol in the host [61], however, we can only speculate about the real function of the SmSP1 LDLa domain.

SmSP2 and SmSP4 form two other separate clades and cluster with trypsin SPs from insect and other invertebrates. Both proteases are characterized by their longer but different N-terminal extensions

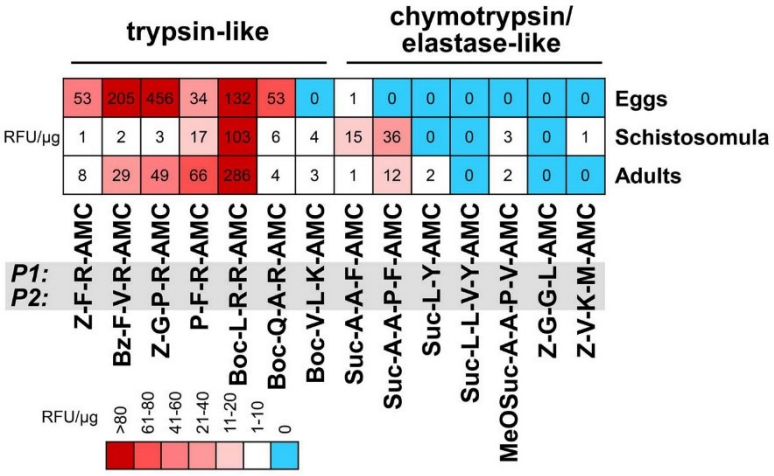

Figure 6. Profiling SP activities in extracts of *S. mansoni* developmental stages. The kinetic assays, performed at pH 8.0, employed 50 µM fluorogenic substrates (P1 and P2 positions are highlighted) that are specific for trypsin- and chymotrypsin/elastase-type proteases. SP activities (sensitive to inhibition by PMSF and Pefabloc SC) are expressed as relative fluorescence units (RFU/s) and normalized to the protein content of extracts. Data are displayed in a heat map.
doi:10.1371/journal.pntd.0002766.g006

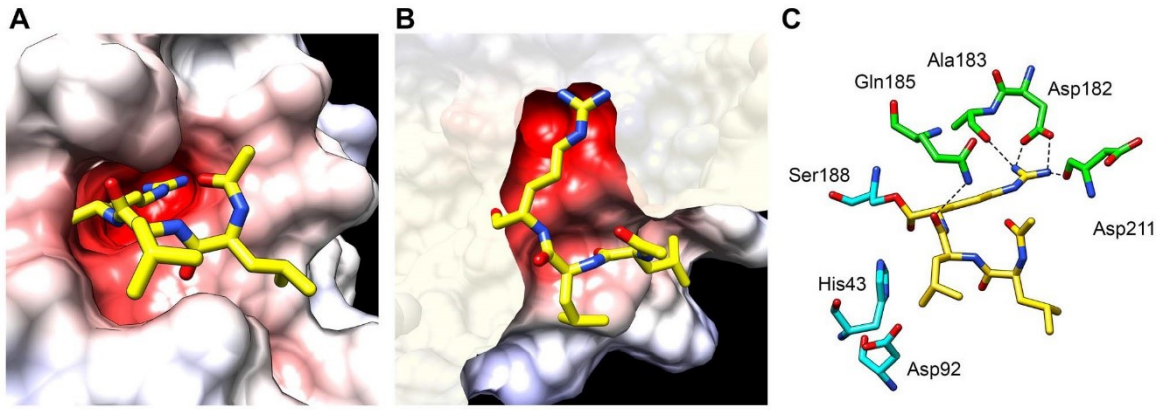

Figure 7. Homology model of the SmSP1 protease domain in complex with leupeptin. The model was built using the template X-ray structure of bovine trypsin in complex with substrate-like inhibitor leupeptin (N-acetyl-L-leucyl-L-leucyl-L-arginal; PDB code 1jrt). (**A**) Surface representation of the SmSP1 active site colored by electrostatic potential (at a scale from −10 kT/e (red) to +10 kT/e (blue)). Carbon atoms of leupeptin are yellow; heteroatoms have a standard color coding (O, red; N, blue). (**B**) The same detail as (A) but viewed from above (the surface display was clipped for a better view). (**C**) Schematic view of the active site residues of SmSP1 (green) forming hydrogen bonds (dashed lines) with leupeptin (yellow). Note the interactions between Asp182 (in the S1 protease subsite) and the basic P1 residue of leupeptin that mimic the S1-P1 salt bridge that is critical for trypsin-like substrate specificity. Catalytic residues (cyan) are shown, including the covalent linkage of leupeptin with the catalytic Ser188.
doi:10.1371/journal.pntd.0002766.g007

that lack homologies to known proteins but which are shared in orthologous SPs from *S. japonicum* [44] and *C. sinensis* [62]. Functions as yet are unknown, however, it is certainly remarkable that SmSP2 is massively expressed in schistosomula and adults (150% and 60% of SmCOX I expression levels, respectively) and, therefore, conceivably contributes significantly to host and/or parasite protein hydrolysis, perhaps in modulating of host physiologic processes [6,12]. The presence also of close orthologs of SmSP2 in *Fasciola gigantica* [63] and *C. sinensis* [62] suggests a general role for SP2 during infection in the mammalian host. The impressive expression levels for SmSP2 are consistent with high levels of SmSP2 expression from microarray [23] and transcriptome data [24]. Also, the expression levels are close to those for the gut-associated, digestive cysteine and aspartic proteases, SmCB1 and SmCD, respectively, for which expression is close to that of SmCOX I [27].

Finally, for SmSP5, phylogenetic analysis identified its relative position in what we term clade number 5. This clade is most closely related to chymotrypsins from invertebrates and comprises SP5 orthologs in *S. japonicum* [44] and *C. sinensis* [62], and the CE genes in *S. mansoni*, *S. haematobium* [20,22], *S. japonicum* [44] and *Schistosomatium douthitti* [20]. Clade 5 is particularly significant for phylogenetic relationship studies of schistosome proteolytic enzymes as it contains sequences that bridge the outlier CE group and other 'more typical' S1 family SPs. Specifically, our previous phylogenetic work [18] had highlighted that the CEs coalesce as a tight group that diverges from other family S1 protease sequences. At that time the SmSP5 sequence was incomplete and not amenable to analysis [18]. The current sequence analysis suggests that SmSP5 and its trematode orthologs are 'a missing link' between the outlier CE group and the common ancestor. CEs initially evolved from chymotrypsin regulatory proteases and may provide an evolutionary advantage in contributing to host invasion [22].

For the SmSP protease domains, we investigated the structure-function relationships using primary structure analysis, homology modeling and protease activity profiling with peptidyl substrates. The sequence alignment shows that all the SmSPs except SmSP5 share a conserved Asp182 residue. This residue defines the specificity for the S1 binding site and drives a strong preference for Arg and Lys residues at the P1 position of protein/peptide substrates, as demonstrated for vertebrate trypsins [47]. The homology model of SmSP1 reveals that the S1 pocket with its critical Asp182 residue has an architecture analogous to vertebrate trypsins. In contrast, the S1 binding pocket of SmSP5 has a Gly182. Also, SmSP5 lacks the disulfide Cys184-Cys212 which is present in the other four SmSPs and known to stabilize the S1 binding site in vertebrate trypsins. Interestingly, this disulfide is also absent in schistosome CEs, which contain non-polar residues (Ile or Leu) at the bottom of the S1 binding pocket resulting in elastase and chymotrypsin-like activities [22].

Consistent with the number of trypsin-like sequences in all of the life-stages studied, major trypsin-like activities could also be measured using peptidyl fluorogenic substrates in eggs, schistosomula and adult extracts. Eggs, in particular, presented the most diverse and active profile compared to adults and schistosomula suggesting they express more than one highly active SP. Schistosomula, in contrast, displayed an activity profile restricted to one substrate, and one might suppose that this activity is in fact due to SmSP2 which was, expressed at higher levels than other SPs as measured by RT-qPCR (see above). Finally, the finding that significant trypsin-like activity was found in the E/S products of the three life stages tested suggests that one or more SmSPs are secreted into the host environment where they may interfere with relevant proteolytic cascades such as blood coagulation, complement or blood pressure regulation [6,12].

In contrast to the trypsin-like activities measured, chymotrypsin/elastase-like activities were absent in eggs, and in schistosomula were at least one order of magnitude weaker. It is possible that the activity in schistosomula is, in whole or part, due to residual CE activity carried forward after mechanical transformation of cercariae and *in vitro* culture of schistosomula. In adults, however, this possibility seems remote and the minor activities measured may be contributed to by SP5.

To conclude, the present study provides a comprehensive phylogenetic, transcriptomic and functional framework illustrating the heretofore unknown complexity of schistosome S1 family SPs,

other than the well-studied CEs [20,22]. The individual enzymes underlying the activities measured remain 'undiscovered country' both in terms of their functional characterization and, not least, their possible contributions to successful parasitism by the schistosome, including at the host-parasite interface.

Supporting Information

Figure S1 Amino acid sequence alignment of three versions of SmSP1. Original description (CAA09691), the *S. mansoni* GeneDB (SchistoDB, Smp_030350) description (both Puerto Rican isolates) and our current version (KF535923) sequenced from a Liberian *S. mansoni* isolate. Sequence variations are highlighted in green, turquoise and grey for KF535923, Smp_030350 and CAA0969, respectively. The CUB, LDLa and trypsin domains are underlined in blue, yellow and red, respectively.
(TIF)

Figure S2 Expression of SmSP1 (CUB, LDLa and protease domains) and SmSP3 (CUB and protease domains) using PCR. Primers were designed to amplify particular domains, partial or whole ORF fragments from cDNA of various *S. mansoni* developmental stages. The lanes are as follows: 1, SmSP1CUB; 2, SmSP1CUB-LDLa; 3, SmSP1trypsin; 4, SmSP1trypsin-LDLa, 5, whole ORF SmSP1CUB-LDLa-trypsin; 6, SmSP3CUB; 7, SmSP3 trypsin and 8, whole ORF SmSP3 CUB-trypsin.
(TIF)

Figure S3 Matrix of amino acid sequence identities used in Figure 2.
(TIF)

Figure S4 Amino acid sequence alignment of three splice versions of SmSP5. The catalytic residues His, Asp and Ser are highlighted.
(TIF)

Figure S5 Comparison of the structures of SmSP1 and bovine trypsin. A stereo image displaying a superimposition of Cα traces of the homology model of SmSP1 (cyan) and the crystal structure of trypsin (PDB code 1JRT; magenta). The catalytic residues are shown as ball and sticks (SmSP1 in green, trypsin in yellow). Disulfide bonds are depicted in blue (SmSP1) and orange (trypsin).
(TIF)

Table S1 List of primers used for RT-qPCR analysis.
(PDF)

Table S2 The list of family S1 proteases (SPs sequences) used for the phylogenetic analysis.
(PDF)

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Author Contributions

Conceived and designed the experiments: MH PF LRA LU PBS MM JD. Performed the experiments: MH PF LRA LU PBS ZF AVP DO JV JD. Analyzed the data: MH PF LRA PBS AVP JV MM CRC JD. Contributed reagents/materials/analysis tools: MH PBS AVP JV JHM MM CRC JD. Wrote the paper: MH JHM MM CRC JD.

References

1. Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J (2006) Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect Dis 6: 411–425.
2. Hall SC, Kehoe EL (1970) Case reports. Prolonged survival of Schistosoma japonicum. Calif Med 113: 75–77.
3. Warren KS (1982) The secret of the immunopathogenesis of schistosomiasis: in vivo models. Immunol Rev 61: 189–213.
4. Abdulla MH, Lim KC, Sajid M, McKerrow JH, Caffrey CR (2007) Schistosomiasis mansoni: novel chemotherapy using a cysteine protease inhibitor. PLoS Med 4: e14.
5. Skelly PJ, Alan Wilson R (2006) Making sense of the schistosome surface. Adv Parasitol 63: 185–284.
6. Da'dara A, Skelly PJ (2011) Manipulation of vascular function by blood flukes? Blood Rev 25: 175–179.
7. Barrett AJ, Rawlings ND, Woessner JF (2004) Handbook of proteolytic enzymes. Amsterdam ; Boston: Elsvier Academic Press.
8. Rawlings ND, Barrett AJ, Bateman A (2012) MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 40: D343–D350.
9. Sajid M, McKerrow JH (2002) Cysteine proteases of parasitic organisms. Mol Biochem Parasitol 120: 1–21.
10. McKerrow JH, Caffrey C, Kelly B, Loke P, Sajid M (2006) Proteases in parasitic diseases. Annu Rev Pathol 1: 497–536.
11. de Oliveira Fraga LA, Lamb EW, Moreno EC, Chatterjee M, Dvorak J, et al. (2010) Rapid induction of IgE responses to a worm cysteine protease during murine pre-patent schistosome infection. BMC Immunol 11: 56.
12. Carvalho WS, Lopes CT, Juliano L, Coelho PM, Cunha-Melo JR, et al. (1998) Purification and partial characterization of kininogenase activity from Schistosoma mansoni adult worms. Parasitology 117 (Pt 4): 311–319.
13. Cocude C, Pierrot C, Cetre C, Fontaine J, Godin C, et al. (1999) Identification of a developmentally regulated Schistosoma mansoni serine protease homologous to mouse plasma kallikrein and human factor I. Parasitology 118 (Pt 4): 389–396.
14. Cocude C, Pierrot C, Cetre C, Godin C, Capron A, et al. (1997) Molecular characterization of a partial sequence encoding a novel Schistosoma mansoni serine protease. Parasitology 115 (Pt 4): 395–402.
15. Doenhoff MJ, Stanley RG, Pryce D, Curtis RC, Parry H, et al. (2003) Identification of a fibrinolytic enzyme in Schistosoma mansoni eggs and modulated blood fibrinogen metabolism in S. mansoni-infected mice. Parasitology 126: 231–234.
16. Kasny M, Mikes L, Hampl V, Dvorak J, Caffrey CR, et al. (2009) Chapter 4. Peptidases of trematodes. Adv Parasitol 69: 205–297.
17. Caffrey CR, McKerrow JH, Salter JP, Sajid M (2004) Blood 'n' guts: an update on schistosome digestive peptidases. Trends Parasitol 20: 241–248.
18. Dvorak J, Mashiyama ST, Braschi S, Sajid M, Knudsen GM, et al. (2008) Differential use of protease families for invasion by schistosome cercariae. Biochimie 90: 345–358.
19. Ingram J, Knudsen G, Lim KC, Hansell E, Sakanari J, et al. (2011) Proteomic Analysis of Human Skin Treated with Larval Schistosome Peptidases Reveals Distinct Invasion Strategies among Species of Blood Flukes. PLoS Negl Trop Dis 5: e1337.
20. Salter JP, Choe Y, Albrecht H, Franklin C, Lim KC, et al. (2002) Cercarial elastase is encoded by a functionally conserved gene family across multiple species of schistosomes. Journal of Biological Chemistry 277: 24618–24624.
21. Schechter I (2005) Mapping of the active site of proteases in the 1960s and rational design of inhibitors/drugs in the 1990s. Curr Protein Pept Sci 6: 501–512.
22. Ingram JR, Rafi SB, Eroy-Reveles AA, Ray M, Lambeth L, et al. (2012) Investigation of the proteolytic functions of an expanded cercarial elastase gene family in Schistosoma mansoni. PLoS Negl Trop Dis 6: e1589.
23. Gobert GN, Tran MH, Moertel L, Mulvenna J, Jones MK, et al. (2010) Transcriptional changes in Schistosoma mansoni during early schistosomula development and in the presence of erythrocytes. PLoS Negl Trop Dis 4: e600.
24. Protasio AV, Dunne DW, Berriman M (2013) Comparative Study of Transcriptome Profiles of Mechanical- and Skin-Transformed Schistosoma mansoni Schistosomula. PLoS Negl Trop Dis 7: e2091.
25. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, et al. (2009) The genome of the blood fluke Schistosoma mansoni. Nature 460: 352–358.
26. Protasio AV, Tsai IJ, Babbage A, Nichol S, Hunt M, et al. (2012) A Systematically Improved High Quality Genome and Transcriptome of the Human Blood Fluke Schistosoma mansoni. PLoS Negl Trop Dis 6: e1455.
27. Dvorak J, Mashiyama ST, Sajid M, Braschi S, Delcroix M, et al. (2009) SmCL3, a gastrodermal cysteine protease of the human blood fluke Schistosoma mansoni. PLoS Negl Trop Dis 3: e449.
28. Colley DG, Wikel SK (1974) Schistosoma mansoni: simplified method for the production of schistosomules. Exp Parasitol 35: 44–51.

29. Basch PF (1981) Cultivation of Schistosoma mansoni in vitro. I. Establishment of cultures from cercariae and development until pairing. J Parasitol 67: 179–185.
30. Stefanic S, Dvorak J, Horn M, Braschi S, Sojka D, et al. (2010) RNA interference in Schistosoma mansoni schistosomula: selectivity, sensitivity and operation for larger-scale screening. PLoS Negl Trop Dis 4: e850.
31. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 365–386.
32. Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. Nat Protoc 1: 1559–1582.
33. Le TH, Blair D, Agatsuma T, Humair PF, Campbell NJ, et al. (2000) Phylogenies inferred from mitochondrial gene orders-a cautionary tale from the parasitic flatworms. Mol Biol Evol 17: 1123–1125.
34. Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30: 3059–3066.
35. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 95–98.
36. Swofford D.L. (2001) PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
37. Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690.
38. Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12: 357–358.
39. Asch HL, Dresden MH (1979) Acidic thiol proteinase activity of Schistosoma mansoni egg extracts. J Parasitol 65: 543–549.
40. Delcroix M, Sajid M, Caffrey CR, Lim KC, Dvorak J, et al. (2006) A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. J Biol Chem 281: 39316–39329.
41. Labute P (2008) The generalized Born/volume integral implicit solvent model: Estimation of the free energy of hydration using London dispersion instead of atomic surface area. Journal of Computational Chemistry 29: 1693–1698.
42. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. Proc Natl Acad Sci U S A 98: 10037–10041.
43. Dolinsky TJ, Czodrowski P, Li H, Nielsen JE, Jensen JH, et al. (2007) PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. Nucleic Acids Res 35: W522–525.
44. Consortium SjGSaFA (2009) The Schistosoma japonicum genome reveals features of host-parasite interplay. Nature 460: 345–351.
45. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8: 785–786.
46. Cohen FE, Gregoret LM, Amiri P, Aldape K, Railey J, et al. (1991) Arresting tissue invasion of a parasite by protease inhibitors chosen with the aid of computer modeling. Biochemistry 30: 11221–11229.
47. Huber R, Bode W (1979) Structural Basis of the Activation, Action and Inhibition of Trypsin. Hoppe-Seylers Zeitschrift Fur Physiologische Chemie 360: 489–489.
48. Bode W, Schwager P (1975) The refined crystal structure of bovine beta-trypsin at 1.8 A resolution. II. Crystallographic refinement, calcium binding site, benzamidine binding site and active site at pH 7.0. J Mol Biol 98: 693–717.
49. Harris JL, Backes BJ, Leonetti F, Mahrus S, Ellman JA, et al. (2000) Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. Proc Natl Acad Sci U S A 97: 7754–7759.
50. Kurinov IV, Harrison RW (1996) Two crystal structures of the leupeptin-trypsin complex. Protein Sci 5: 752–758.
51. Tort J, Brindley PJ, Knox D, Wolfe KH, Dalton JP (1999) Proteinases and associated genes of parasitic helminths. Adv Parasitol 43: 161–266.
52. Tsang VC, Damian RT (1977) Demonstration and mode of action of an inhibitor for activated Hageman factor (factor XIIa) of the intrinsic blood coagulation pathway from Schistosoma mansoni. Blood 49: 619–633.
53. Foster CB, Flanigan TP, DeStigter KK, Blanton R, Dumenco LL, et al. (1992) Inhibition of the activation of Hageman factor (factor XII) by extracts of Schistosoma mansoni. J Lab Clin Med 120: 735–739.
54. El-Bassiouni NE, El Bassiouny AE, Hussein NA, El-Sayed HH, Ibrahim IM, et al. (1998) The coagulation profile in hepatosplenic schistosomiasis. Blood Coagul Fibrinolysis 9: 189–194.
55. Page MJ, Di Cera E (2008) Serine peptidases: classification, structure and function. Cell Mol Life Sci 65: 1220–1236.
56. Bork P, Beckmann G (1993) The CUB domain. A widespread module in developmentally regulated proteins. J Mol Biol 231: 539–545.
57. Mourao MM, Dinguirard N, Franco GR, Yoshino TP (2009) Phenotypic screen of early-developing larvae of the blood fluke, schistosoma mansoni, using RNA interference. PLoS Negl Trop Dis 3: e502.
58. Rinaldi G, Morales ME, Alrefaei YN, Cancela M, Castillo E, et al. (2009) RNA interference targeting leucine aminopeptidase blocks hatching of Schistosoma mansoni eggs. Mol Biochem Parasitol 167: 118–126.
59. Fan J, Gan X, Yang W, Shen L, McManus DP, et al. (2003) A Schistosoma japonicum very low-density lipoprotein-binding protein. Int J Biochem Cell Biol 35: 1436–1451.
60. Meyer F, Meyer H, Bueding E (1970) Lipid metabolism in the parasitic and free-living flatworms, Schistosoma mansoni and Dugesia dorotocephala. Biochim Biophys Acta 210: 257–266.
61. Stanley RG, Jackson CL, Griffiths K, Doenhoff MJ (2009) Effects of Schistosoma mansoni worms and eggs on circulating cholesterol and liver lipids in mice. Atherosclerosis 207: 131–138.
62. Wang X, Chen W, Huang Y, Sun J, Men J, et al. (2011) The draft genome of the carcinogenic human liver fluke Clonorchis sinensis. Genome Biol 12: R107.
63. Young ND, Jex AR, Cantacessi C, Hall RS, Campbell BE, et al. (2011) A portrait of the transcriptome of the neglected trematode, Fasciola gigantica–biological and biotechnological implications. PLoS Negl Trop Dis 5: e1004.

Publikace č. 3

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

SmSP2: A serine protease secreted by the blood fluke pathogen *Schistosoma mansoni* with anti-hemostatic properties

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Abstract

Background

Serine proteases are important virulence factors for many pathogens. Recently, we discovered a group of trypsin-like serine proteases with domain organization unique to flatworm parasites and containing a thrombospondin type 1 repeat (TSR-1). These proteases are recognized as antigens during host infection and may prove useful as anthelminthic vaccines, however their molecular characteristics are under-studied. Here, we characterize the structural and proteolytic attributes of serine protease 2 (SmSP2) from *Schistosoma mansoni*, one of the major species responsible for the tropical infectious disease, schistosomiasis.

Methodology/Principal findings

SmSP2 comprises three domains: a histidine stretch, TSR-1 and a serine protease domain. The cleavage specificity of recombinant SmSP2 was determined using positional scanning and multiplex combinatorial libraries and the determinants of specificity were identified with 3D homology models, demonstrating a trypsin-like endopeptidase mode of action. SmSP2 displayed restricted proteolysis on protein substrates. It activated tissue plasminogen activator and plasminogen as key components of the fibrinolytic system, and released the vasoregulatory peptide, kinin, from kininogen. SmSP2 was detected in the surface tegument, esophageal glands and reproductive organs of the adult parasite by immunofluorescence microscopy, and in the excretory/secretory products by immunoblotting.

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Conclusions/Significance

The data suggest that SmSP2 is secreted, functions at the host-parasite interface and contributes to the survival of the parasite by manipulating host vasodilatation and fibrinolysis. SmSP2 may be, therefore, a potential target for anti-schistosomal therapy.

Author summary

Schistosomiasis (bilharziasis) is a global parasitic infection with more than 240 million people infected. It is caused by *Schistosoma* flatworms that live in the bloodstream. Current treatment relies on one drug, and no effective vaccine has yet been developed. Proteolytic enzymes (proteases) help the parasite survive in the mammalian host, allowing the schistosome to invade, feed, grow, reproduce and, manipulate the immune system. Thus, proteases are considered potential drug and vaccine targets. We previously described, and herein, investigate at the protein level, SmSP2, the major serine protease produced by the blood-dwelling stages of *S. mansoni*. We show that SmSP2 is secreted by the parasite and effectively processes host blood bioactive peptides and proteins that are involved in fibrinolysis and regulation of vascular tone. We propose, therefore, that SmSP2 modulates host hemostasis to promote parasite infection and survival. Thus, SmSP2 and similar proteins in other parasitic flatworms represent potential targets for novel drug or vaccine interventions.

Introduction

Schistosomiasis (bilharziasis) is a chronic infectious disease caused by a trematode blood flukes of the genus *Schistosoma*. A public health problem in 74 developing countries, the parasite infects over 240 million people with up to 700 million people at risk [1, 2]. Adult schistosomes can reside for decades in the host vascular system as male-female pairs producing hundreds of eggs per day [3]. Morbidity arises from immuno-pathological reactions to and entrapment of schistosome eggs in various tissues [4]. In the absence of a vaccine, treatment and control of schistosomiasis relies on a single drug, praziquantel (PZQ) [5, 6]. During its complex life cycle, the parasite survives in various environments by presenting or releasing bioactive molecules that aid survival and modulate host physiology [7–10]. Disruption of these potential mechanisms by specific drugs or vaccines may provide therapeutic benefits.

Proteolytic enzymes (proteases) of schistosomes [11] are attractive drug targets as they frequently operate at the host–parasite interface, and facilitating parasite invasion, migration, nutrition and immune evasion [12–14]. Most studies on schistosome proteases, to date, have focused on cysteine and aspartic proteases that contribute to the digestion of the blood meal [15, 16]. Among them, the cathepsin B1 of *S. mansoni* (SmCB1) has been validated in a murine model of schistosomiasis as a chemotherapeutic target [17], and small molecule inhibitors of SmCB1 are under investigation as potential drug leads [18–20].

Schistosome serine proteases (SPs) are less studied with the exception of cercarial elastase, which facilitates penetration of the human host by infective larvae [21, 22]. Recently, we uncovered a repertoire of *S. mansoni* trypsin- and chymotrypsin-type S1 family serine proteases (SmSPs) by employing a series of genomic, transcriptomic, proteolytic and phylogenetic investigational strategies [23]. Among these, SmSP2 is the most abundantly expressed in blood-dwelling stages [23]. Interestingly, SmSP2 ortholog (mastin) was identified as potential

vaccine targets in *Schistosoma haematobium* based on IgG1 immune response of individuals with drug-induced resistance [24].

In this study, we report the first detailed biochemical and enzymatic characterization of SmSP2. This enzyme processes several proteins and peptides that are involved in host proteolytic cascades, *i.e.*, blood coagulation, fibrinolysis and blood pressure regulation, and, thus, may interfere with critical vascular hemostatic processes. Accordingly, SmSP2 may be a target for novel anti-schistosomal therapeutics.

Materials and methods

Ethics statement

Research with experimental animals was performed in accordance with the animal welfare laws of the Czech Republic and in accordance with European regulations for transport, housing and care of laboratory animals (Directive 2010/63/EU on the protection of animals used for scientific purposes). The project of experiments including the use of experimental animals for present study was approved by Ministry of education, youth and sports of the Czech Republic (approval number MSMT—7063/2017-2). All the animals used in the study were maintained by certified person (certificate number CZ 02627) in specifically accredited laboratories of Institute of Immunology and Microbiology of the First Faculty of Medicine, Charles University and the General University Hospital in Prague (accreditation number 70030/2013-MZE-17214), both issued by the Ministry of Agriculture of the Czech Republic.

Schistosome material

Schistosoma mansoni (a Puerto Rican isolate) was routinely maintained in the laboratory by cycling between the intermediate snail host, *Biomphalaria glabrata*, and outbred ICR mice as definitive hosts. Infective larvae (cercariae) were released after light stimulation from infected snails placed in fresh water. Adult mice were infected by immersing the feet and tails for 45 min in 50 ml of water containing approximately 500 cercariae. Six weeks post infection, mice were euthanized by an intra-peritoneal injection of ketamine (Narkamon 5% - 1.2 mL/kg bw) and xylazine (Rometar 2% - 0.6 mL/kg bw) and the worms recovered from the portal vein by transcardial perfusion with RPMI 1640 medium as described previously [25–27]. Newly transformed schistosomula (NTS) were prepared by mechanically transforming cercariae and cultivated in a Basch Medium 169 [28] containing 5% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin for 5 days at 37˚C under a 5% CO2 atmosphere.

Preparation of schistosome extract, excretory/secretory products collection and purification of native SmSP2

Soluble protein extracts (1–3 mg protein/mL) from *S. mansoni* adults were prepared by homogenization in 50 mM Tris-HCl, pH 8.0, containing 1% CHAPS, 1 mM EDTA, 1 μM pepstatin and 1 μM E-64 on an ice bath. The extract was cleared by centrifugation (16,000 *g* at 4˚C for 10 min), ultra-filtered using a 0.22 μm Ultrafree-MC device (Millipore) and stored at -80˚C. Excretory/secretory products (ESP) of adult worms were collected as described previously [29]. Specifically, fifty pairs of adult worms were washed five times in Basch medium 169 containing 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 1% Fungizone (Gibco), incubated for 1h at 37˚C under a 5% CO_2 atmosphere, washed five times and then incubated overnight at 37˚C in 5% CO_2 in the above medium supplemented with 5% fetal calf serum but in the absence of Fungizone. Parasites were washed three times in the above medium and then washed 10 times in M-199 medium containing 100 U/ml penicillin and

100 μg/ml streptomycin, but without serum. Adults were evenly distributed in 5 ml of the same medium in a 6-well cultivation dish and incubated for 16 h at 37˚C in 5% CO_2. Medium containing ESP was removed, filtered over an Ultrafree-MC 0.22 μm filter (Millipore), buffer exchanged into ice-cold PBS, concentrated to 2 mL using Amicon Ultracel-10K filters (Millipore) and aliquots stored at -80˚C.

Native SmSP2 was purified from the adult schistosome extract using Ni^{2+} chelating chromatography (Hi-Trap IMAC FF column, GE Healthcare Life Sciences) under native conditions. The bound material was eluted using a 0.5 M imidazole and the purified proteins analyzed by immunoblotting with anti-SmSP2 IgG.

Expression and purification of recombinant SmSP2 in *Pichia pastoris*

The full-length SmSP2 gene (GenBank KF510120; GeneDB Smp_002150) without the N-terminal signal sequence predicted by SignalP [30] was codon-optimized for expression in *P. pastoris* and cloned into the pUC57 vector (GenScript) with an incorporated C-terminal His-tag (GPHHHHHH). The SmSP2 protease domain (residues 201 to 501, Fig 1) containing a short N-terminal propeptide (residues 183 to 200, Fig 1) was prepared by PCR amplification of the synthetic SmSP2 gene using the forward primer, 5´-AAGAGAGGCTGAAGC**TGCA**AACTT GACAAACACCTGTGGTATCAG-3´ that contained a Pst I restriction site, and the reverse primer, 5´-GGCCACGTGAATTCCTTAGTGATGGTGATGGTGATGAGGACC-3. Both primers contained 15 nucleotide extensions (underlined) homologous to the ends of Pst I-linearized pPICZαB vector (Thermo Fisher). The PCR product was cloned into this expression vector using the In-Fusion HD Cloning Kit according to manufacturer protocol (Clontech) and verified by DNA sequencing. Transformation of *P. pastoris* X-33 cells (Thermo Fisher) and protein expression were carried out as described previously [31, 32].

The yeast medium containing recombinant SmSP2 was centrifuged (3,000 *g* for 10 min), and the supernatant filtered (0.45 μm), lyophilized and dissolved in 20 mM MES buffer, pH 6.0 (to 10% of the original volume). The solution was then desalted over a Sephadex G-25 column equilibrated with the same buffer. SmSP2 was purified using chromatography on a HiTrap Benzamidine FF column (GE Healthcare Life Sciences) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, and eluted with 50 mM glycine, pH 3.0. The pH of the eluted fractions containing SmSP2 was adjusted to 6.0 by addition of 1M Tris-HCl, pH 8.0, and enzyme fractions were stored in -80˚C. The purification process was monitored with a kinetic activity assay using the fluorogenic substrate, P-F-R-AMC (AMC, 7-amino-4-methylcoumarin), and by SDS-PAGE and Western blotting.

Expression, refolding, purification of recombinant SmSP2 in *E. coli*

The Champion pET directional expression kit (Thermo Fisher) was selected for bacterial expression of the SmSP2 protease domain (residues 201–501) that contained a short N-terminal propeptide (residues 183 to 200, Fig 1). The 957 bp ORF was PCR amplified from a synthetic SmSP2 gene using specific forward (5´- caccATGAATCTAACTAATACATGTGGAA TACGTAAATCA-3´) and reverse (5´- AAATATTTTTGTTGCTAATTGTTTTGATATCC AA-3´) primers. The PCR product was cloned into the expression vector pET100/D-TOPO (Thermo Fisher) incorporating an N-terminal His tag and verified by DNA sequencing. Recombinant SmSP2 was produced in *E. coli* BL21(DE3) (Thermo Fisher), purified from inclusion bodies using Ni^{2+} chelating chromatography under denaturing conditions and the purified protein was refolded using the dialysis protocol described in [33].

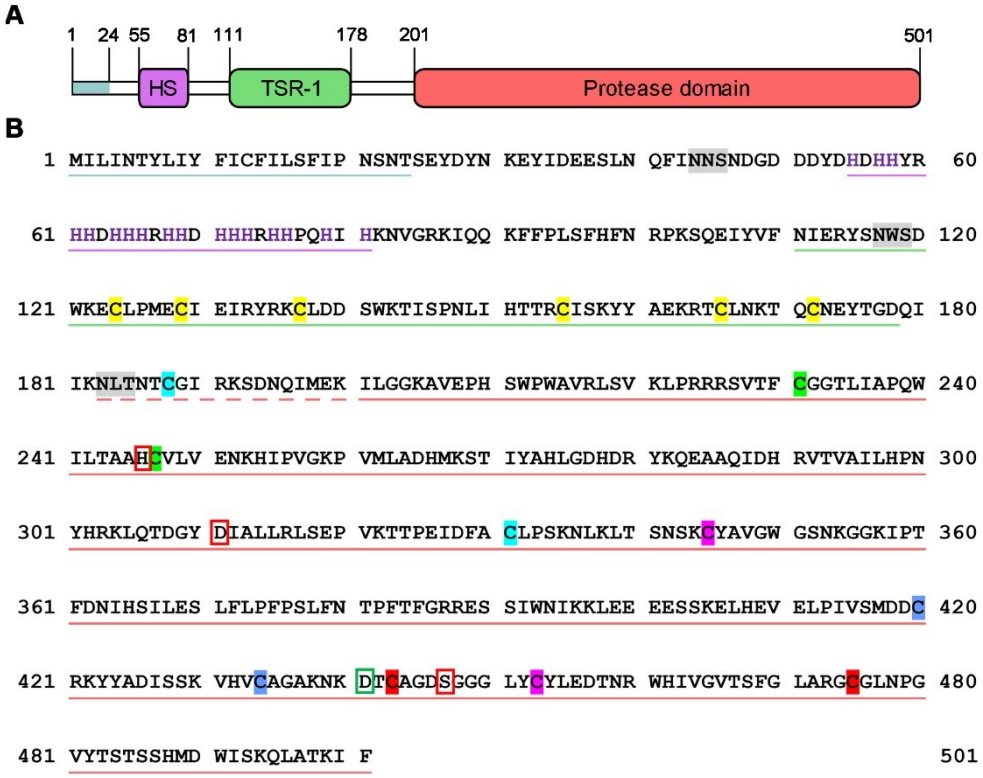

Fig 1. Domain organization and amino acid sequence of SmSP2. (A) Schematic diagram of the domain layout. The N-terminal signal peptide, "His stretch' (HS), thrombospondin type 1 repeat (TSR-1) and S1 family protease domains are depicted in blue, purple, green, and red, respectively. Amino acid residue numbers are indicated. (B) The amino acid sequence of SmSP2 with the various domains color-coded by underlining as in (A). Predicted N-glycosylation sites are highlighted in grey, and His residues in the His stretch are in purple. The catalytic residues, His246, Asp311 and Ser447 are red-boxed; and Asp441 in the S1 subsite that accounts for trypsin-like activity is green-boxed. Cys residues of the protease domain that are predicted to form a disulfide are indicated by the same color; Cys residues of the TSR-1 domain are colored yellow. The propeptide is underlined with dashed red line.

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Preparation of antibodies and immunoblotting

Specific polyclonal antibodies were generated in rabbits (Moravian Biotechnology) against the bacterially-expressed SmSP2 protease domain. Antigen (50 μg in Freund's incomplete adjuvant) was administered three times each three weeks apart. IgG was isolated from the serum by affinity chromatography with a HiTrap Protein A column (GE Healthcare Life Sciences) according to the manufacturer's protocol. Immunoselection of SmSP2-specific IgG was carried out using a standard methodology [34]. Briefly, 800 μg of recombinant SmSP2 expressed in *E. coli* was resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Excised strips containing SmSP2 were blocked with 3% BSA in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.1% Tween (TTBS) for 1 h, washed with TTBS, and incubated with the protein-A purified anti-SmSP2 IgG overnight at 4˚C. After washing, bound antibodies were eluted with 100 mM glycine, pH 2.5 and the elution was immediately adjusted to pH 7.5 using 1 M Tris-HCl.

For immunoblotting, adult schistosome homogenate (30 μg protein) and rSmSP2 (1 μg) were resolved by SDS-PAGE (4–12% NuPAGE gel, Thermo Fisher) under reducing conditions and transferred onto a PVDF membrane. The membrane was blocked for 1 h with 3% BSA in TTBS, washed with TTBS and incubated overnight with anti-SmSP2 polyclonal IgG diluted 1:200 in TTBS. After washing with TTBS, the membrane was incubated for 1 h with goat horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) at a dilution of 1:10,000. After washing with TTBS, the membrane was developed with Luminata Crescendo Western HRP substrate (Merck) and imaged using an ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences).

Immunofluorescence microscopy

Adult *S. mansoni* males and females were carefully separated over ice, washed three times in 100 mM sodium phosphate, pH 7.4, containing 154 mM NaCl (PBS), fixed in Bouin's solution (Sigma-Aldrich) for 45 min at 25˚C, and embedded in paraffin blocks. Sections (6–8 μm each) were deparaffinized in xylol, rehydrated in an ethanol series of decreasing ethanol concentration and boiled in a water bath in 10 mM sodium citrate, pH 6.0, for 15 min. Cooled sections were rinsed with PBS, permeabilized in PBS containing 0.25% Triton X-100 (PBS/Triton) for 20 min, and blocked overnight at 4˚C with 2% BSA in PBS/Triton. After washing in PBS/Triton, the sections were incubated for 60 min at 4˚C with rabbit polyclonal anti-SmSP2 IgG diluted 1:25 in PBS/Triton containing 1% BSA. Sections were then washed three times in PBS/Triton and incubated for 30 min at 25˚C with anti-rabbit IgG Alexa 647-conjugated secondary antibody (Thermo Fisher) diluted 1:500 in PBS/Triton. After washing with PBS/Triton, the sections were mounted in Prolong Diamond antifade reagent containing DAPI (Thermo Fischer). The fluorescence was visualized using an Olympus IX83 microscope equipped with PCO edge 5.5 camera and CoolLED pE-4000 LED illumination system. DAPI signal was detected using excitation diode 365 nm and emission filter 440/40 nm, Alexa 647 using diode 635 nm and emission filter 700/75. Appropriate lightning settings were determined using control slides probed with pre-immune serum to define the background signal threshold. Image stacks of optical sections were processed using the Fiji software.

Active site labeling of SmSP2

Recombinant SmSP2 expressed in *P. pastoris* (1 μg) was incubated 1 h at 37˚C with 1 μM activity-based probe BoRC [35] in 50 mM Tris-HCl, pH 8.0. The competitive labeling reaction was also performed after treatment of SmSP2 for 15 min at 37˚C with 1 mM serine protease inhibitor Pefabloc SC (Sigma-Aldrich). The labeled SmSP2 was precipitated with acetone, resolved by SDS-PAGE, and visualized in-gel using a Typhoon 9410 imager (GE Healthcare) as described previously [36].

Kinetic SmSP2 activity and inhibition assays

SmSP2 activity was measured in continuous kinetic assays using the following peptidyl fluorogenic AMC substrates (Bachem): Z-F-R-AMC (Z, Benzyloxycarbonyl), Bz-F-V-R-AMC (Bz, Benzoyl), P-F-R-AMC, Boc-L-R-R-AMC (Boc, t-Butyloxycarbonyl), Boc-Q-A-R-AMC, Boc-V-L-K-AMC, Suc-A-A-F-AMC (Suc, Succinyl), Suc-A-A-P-F-AMC, MeOSuc-A-A-P-V-AMC (MeOSuc, 3-Methoxysuccinyl), Z-G-G-L-AMC, Z-V-K-M-AMC, Boc-L-G-R-AMC, Z-V-V-R-AMC, V-P-R-AMC, Z-R-R-AMC, R-R-AMC, R-AMC and Boc-L-L-V-Y-AMC. Assays were performed at 37˚C in white 96-well microplates in a total volume of 100 μl. Recombinant SmSP2 expressed in *P. pastoris* (10 ng) was pre-incubated for 10 min at 37˚C in 80 μl of 0.1 M Tris-HCl, pH 8.0. The reaction was initiated by adding 20 μl of substrate

Table 1. Sensitivity of rSmSP2 to protease inhibitors.

Inhibitor[a]	Target protease[b]	Concentration (μM)	Inhibition (%)[c]
Pefabloc SC	SP	1000	100
Benzamidine	SP	1000	100
3,4-dichlorcoumarin	SP	100	71.4 ± 3.3
Antipain	SP, CP	20	100
Leupeptin	SP, CP	20	100
BPTI (Aprotinin)	SP	10	87.0 ± 2.6
STI	SP	10	91.8 ± 0.9
α-1-antichymotrypsin	SP	1	13.2 ± 2.4
α-1-antitrypsin	SP	1	79.0 ± 0.9
Antithrombin III	SP	1	99.1 ± 0.3
PAI-1	SP	1	100
E-64	CP	10	7.2 ± 0.9
Pepstatin A	AP	1	0
EDTA	MP	1000	3.2 ± 0.9
Bestatin	MP	1	4.5 ± 2.0
Phenanthroline	MP	1000	26.9 ± 4.7
α-2-macroglobulin	all types	0.1	61.6 ± 0.4

[a] Abbreviations: BPTI (bovine pancreatic trypsin inhibitor), STI (soybean trypsin inhibitor), E64 (trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane), PAI-1 (plasminogen activator inhibitor-1).

[b] The target proteases are classified based on catalytic type into aspartic (AP), cysteine (CP), serine (SP) proteases and metalloproteases (MP).

[c] rSmSP2 was pre-incubated with the given inhibitor and remaining activity was measured in a kinetic assay with the fluorogenic substrate P-F-R-AMC. The mean values ± S.D. of three replicates are expressed as the percentage inhibition relative to uninhibited controls.

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solution (50 μM final concentration) in the same buffer. Proteolytic activity was measured continuously in an Infinite M1000 microplate reader (Tecan) at excitation and emission wavelengths of 360 and 465 nm, respectively. The pH activity profile was determined in 50 mM citrate, 100mM phosphate (pH range 3.0–7.5), and 0.1M glycine (pH range 8.0–11.0) using the P-F-R-AMC substrate. The pH profile of SmSP2 activity in schistosome homogenates (1–5 μg of protein) was measured analogously, but in the presence of 10 μM E-64 and 1 mM EDTA to prevent undesired proteolysis by cysteine proteases and metalloproteases, respectively. Protease activity was expressed as the remaining portion that was sensitive to 1 mM Pefabloc SC. For inhibition measurements, inhibitors were added to the 80 μL pre-incubation solution at the final concentrations listed in Table 1. After 10 min at 37˚C, reactions were initiated by the addition of substrate. For pH stability determinations, SmSP2 was incubated in 50 mM citrate, 100mM phosphate (pH range from 3.0 to 7.0) or 0.1M glycine (pH range from 8.0 to 11.0). After 1, 4 and 20 h, aliquots containing 10 ng SmSP2 were taken and SmSP2 activity was measured using P-F-R-AMC as described above. All measurements were done in triplicate.

Interaction of SmSP2 with protein substrates

Recombinant SmSP2 expressed in *P. pastoris* (300 ng) was incubated at 37˚C with 1–20 μg of human plasminogen (hPLG, R&D Systems), high molecular weight human plasma kininogen (HMWK; Merck), human tissue plasminogen activator (tPA), human serum albumin (HSA), human hemoglobin, calf collagen type I, human fibronectin and rabbit immunoglobulin G (all Sigma-Aldrich) in 25 μL 100 mM Tris-HCl, pH 8.0. After incubation (between 1 and 48 h), the reaction was stopped by adding Pefabloc SC (final concentration 1 mM) and 20 μl of the

reaction was resolved by SDS-PAGE (4-12% Nupage gel) and stained with Coomassie Brilliant Blue G250. In control experiments, protein was incubated in the absence of SmSP2 and analyzed under identical conditions. Processing products generated during HMWK degradation were identified by mass spectrometry, the reaction mixture was analyzed using LC-MS/MS as described previously [32, 37]. To analyze the activation of hPLG and tPA by SmSP2, aliquots of the reaction mixtures containing 100 ng of hPLG or tPA were withdrawn at different time intervals and activity was measured in a kinetic assay using 50 μM Boc-V-L-K-AMC or Z-G-G-R-AMC, respectively, in 100 mM Tris-HCl, pH 7.5, containing 100 mM NaCl. Proteolytic activity was measured continuously as described above. In control experiments, SmSP2, hPLG or tPA alone were analyzed under identical conditions.

Hydrolysis of peptide hormones by SmSP2

Extended bradykinin (Ac-SLMKRPPGFSPFRSSR-amide, Ac, acetyl) was synthesized as a peptidyl amide by Fmoc solid phase chemistry in an ABI 433A peptide synthesizer (Applied Biosystems), as described previously [19, 38]. Recombinant SmSP2 expressed in *P. pastoris* (200 ng) was incubated at 37˚C for 1 to 16 h with 25 nmol of bradykinin, lysyl-bradikinin (kallidin), vasopresin (all Sigma-Aldrich) or extended bradykinin in 0.1 M Tris-HCl, pH 8.0, in a total volume of 50 μL. The reaction was stopped by adding TFA to a final concentration of 1%. The resulting fragments were purified by reverse-phase HPLC using a Luna C18 column (25 x 0.46 cm, Phenomenex) and the TFA/acetonitrile system, and characterized by MS/MS [32, 37].

Hydrolysis of peptides by cultured schistosomes

Adult worms were washed and treated as described above (section Preparation of schistosome extract, excretory/secretory products collection and purification of native SmSP2). Five adult schistosome pairs were then placed into clear, 24-well, flat-bottom plates (Costar) containing 500 μL Basch Medium 199 [28], supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (without fetal bovine serum). Human vasopressin or extended bradykinin (10 μM) was added and the incubation continued for 16 h at 37˚C under a 5% CO2 atmosphere. In control experiments, the peptides were incubated in the same system but in the absence of schistosomes. After incubation, the samples were ZipTiped and the resulting fragments were analyzed using MALDI-TOF performed on an UltrafleXtreme (Bruker Daltonik) operated in reflectron mode with an acceleration voltage of 25 kV and a pulsed ion extraction of 120 ns. Desorption and ionization were achieved using a Smartbeam II laser. α-Cyano-4-hydroxycinnamic acid was used as a matrix. The data were acquired from m/z 220 to 3700 and analyzed with the FlexAnalysis 3.3 software (Bruker Daltonik). The mass spectra were externally calibrated using a Peptide Calibration Standard I (Bruker Daltonik) and averaged from 3000 laser shots.

Subsite profiling of SmSP2 by a positional scanning synthetic combinatorial library (PS-SCL) and by multiplex cleavage assays

Synthesis of the PS-SCL has been previously described [39]. The assays were carried out in black 96-well microplates in 0.1 M Tris-HCl, pH 8.0, containing 0.01% Tween 20 and initiated by addition of recombinant SmSP2 (10 ng). Release of 7-amino-4-carbamoylmethylcoumarin (ACC) was measured in an SpectraMax Gemini fluorescence spectrometer (Molecular Devices) with excitation and emission wavelengths set to 380 and 460 nm, respectively.

The Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) assay was performed as previously described [29]. SmSP2, human plasma kallikrein (Sigma-Aldrich) and bovine trypsin Sigma-Aldrich) (all 17 nM) were incubated in triplicate with a mixture of 228 synthetic

tetradecapeptides (0.5 uM each) in 10 mM Tris-HCl, pH 8.0. After 15, 60, and 240 minutes, 20 μL aliquots were removed, quenched with 6.4 M guanidinium chloride, immediately frozen at -80˚C. Control reactions were performed by treating enzymes with guanidinium chloride prior to peptide exposure. Samples were acidified to < pH 3.0 with 1% formic acid, desalted with C18 LTS tips (Rainin), and injected into a Q Exactive Mass Spectrometer (Thermo) equipped with an Ultimate 3000 HPLC (Thermo). Peptides separated by reverse phase chromatography on a C18 column (1.7 um bead size, 75 um x 25 cm, heated to 65˚C) at a flow rate of 300 nL min^{-1} using a 55-min. linear gradient from 5% B to 30% B, with solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile. Survey scans were recorded over a 150–2000 m/z range at 70000 resolutions (AGC target 1×106, 75 ms maximum). MS/MS was performed in data-dependent acquisition mode with HCD fragmentation (30 normalized collision energy) on the 10 most intense precursor ions at 17500 resolutions (AGC target 5×104, 120 ms maximum, dynamic exclusion 15 s).

Peak integration and peptide identification were performed using Peaks software (Bioinformatics Solutions Inc.). Quantification data are normalized by LOWESS and filtered by 0.3 peptide quality. Missing and zero values are imputed with random numbers in the range of the average of smallest 5% of the data ± sd. Differences between each time point and no-enzyme control were analyzed for statistical significance by multiple t-test. When compared to the control reaction, peptide cleavage products with >10-fold change in peak area intensity and p-value <0.05 were identified and the peptide sequence corresponding to the P4 to P4' subsite positions were used to make IceLogo frequency plots [40]. Mass spectrometry data and results can be accessed here: ftp://massive.ucsd.edu/MSV000081747.

Molecular modeling of SmSP2

A spatial model of the SmSP2 protease domain was constructed by homology modeling, as described previously [37]. Briefly, the X-ray structures of human mannan-binding lectin serine protease 1 (MASP-1) and bovine trypsin ((Protein Data Bank (PDB) entries: 3GOV and 1JRT, respectively) were used as templates. The homology module generated by the MOE program (Chemical Computing Group, Canada) was used to model the SmSP2 structure. The inhibitor conformation was refined by applying the LigX module of the MOE and the final binding mode of the inhibitor was selected by the best-fit model based on the London dG scoring function and the generalized Born method [37]. Molecular images were generated with UCSF Chimera (http://www.cgl.ucsf.edu/chimera/).

Results

SmSP2 is a S1 family protease with a unique domain organization

The SmSP2 open reading frame consists of 1,506 bp that encode a protein of 501 amino acid residues with a theoretical molecular mass of 58 kDa. The predicted signal sequence is 24 residues long and the amino acid sequence contains three potential N-glycosylation sites (Fig 1). Based on sequence homology analysis, we describe SmSP2 as a multi-domain protein made up of an N-terminal region (residues 25 to 110), a thrombospondin-1 type 1 repeat (TSR-1) domain (residues 111 to 178) and a S1 family serine protease domain at the C-terminus (residues 201 to 501). The N-terminal region lacks significant similarity with other published protein sequences in databases. The striking feature of this region is the presence of a stretch of histidine residues (the 'His stretch'—residues 55 to 81) which suggests that SmSP2 may bind metal ions.

The TSR-1 domain has been identified in multiple protein families and occurs in more than 40 human proteins, *e. g.*, thrombospondins, ADAMTS (A Disintegrin And

Metalloproteinase with Thrombospondin Motif), properdin and some complement factors [41]. It is known to mediate cell adhesion, protein-protein interactions, glycosaminoglycan binding and inhibit angiogenesis [42, 43]. The TSR-1 of SmSP2 consists of about 60 residues and its sequence contains all of the important conserved features of TSR-1 domains (S1 Fig): a cysteine residue pattern and conservation of tryptophan and arginine residues forming so called W and R layers [44].

The catalytic protease domain of SmSP2 belongs to the S1 family of serine proteases and has about 30% identity with other members of this family (S2 Fig). The protease domain of SmSP2 possesses a catalytic triad of His246, Asp311 and Ser447 (corresponding to amino acid residues 57, 102 and 195 by standard chymotrypsinogen numbering), which is typical of S1 family proteases. In addition, the amino acids surrounding the catalytic-triad residues have the highest sequence identity with other S1 family enzymes (S2 Fig). Cysteine residues in the catalytic domain form four conserved disulfide bonds that can be predicted by the alignment with the solved crystal structures of S1 family proteases (S2 Fig). Moreover, an additional cysteine residue, Cys311, is likely to form a disulfide bond with a Cys188 in the N-terminal region. An analogous disulfide bond is described, for example, in bovine chymotrypsinogen, human plasmin, urokinase (uPA), tissue plasminogen activators (tPA) and MASP-1. In bovine trypsin, Asp189 is located at the bottom of the S1 binding site and determines the trypsin-like specificity for substrates with Arg/Lys in the P1 position [45]. This residue is conserved in the sequence of SmSP2 (Asp441). When compared to bovine chymotrypsin, the S1 family holotype protease [46], SmSP2 has three insertions (S2 Fig) located between residues 222 and 226 (insertion-222), 251 and 268 (insertion-251), and between residues 358 and 400 (insertion-358). Whereas protein sequences corresponding to the short insertions-222 and -251 are found in uPA/tPA and MASP1, respectively, the long insertion-358 is unique to SmSP2.

SmSP2 orthologs are found in: (i) other schistosome species—*S. japonicum* (GenBank: AAW24683.1) and *S. haematobium* (XP_012796372.1) sharing 80% and 78% sequence identity, respectively; (ii) other trematodes, including, *Fasciola hepatica* (identified in the transcriptome database [47] - 53% identity), *Opisthorchis viverrini* (XP_009167273.1–49% identity), *Clonorchis sinensis* (GAA32831.2–47% identity); and (iii) the sequences of cestodes such as *Hymenolepis microstoma* (CDS25513.1), *Echinococcus multilocularis* (CDI97096.1), *Echinococcus granulosus* (CDI97096.1), and *Taenia solium* (ADP89566.1) sharing 27–39% identities (S3 Fig). All of the ortholog sequences contain the TSR-1 and protease domains, however, they differ in the N-terminal region in length and the presence of the His stretch that is unique to trematodes. Moreover, the insertion-358 in the cestode sequences is shorter and contains two additional Cys residues that might potentially form a disulfide stabilizing this structure (S3 Fig). To conclude, SmSP2 and its orthologs are serine proteases with unique domain organization found exclusively in the phylum Platyhelminthes.

Homology model of SmSP2 reveals the trypsin-like active site pocket shielded by additional loops

A spatial model of the SmSP2 protease domain was constructed by homology modelling to provide a structural framework to analyze structure-activity relationships. The X-ray structure of bovine trypsin (PDB code 1JRT) and human MASP-1 (PDB code 3GOV) were used as templates. Fig 2A shows that the SmSP2 protease domain has the conserved architecture of S1 family proteases which consists of two six-stranded β-barrel domains packed against each other. The catalytic amino acid residues, His246, Asp311 and Ser447 are located at the junction between these β-barrel domains. The major sequence insertions in SmSP2 compared to trypsin are located at surface-exposed loops surrounding the substrate binding region (Fig 2B).

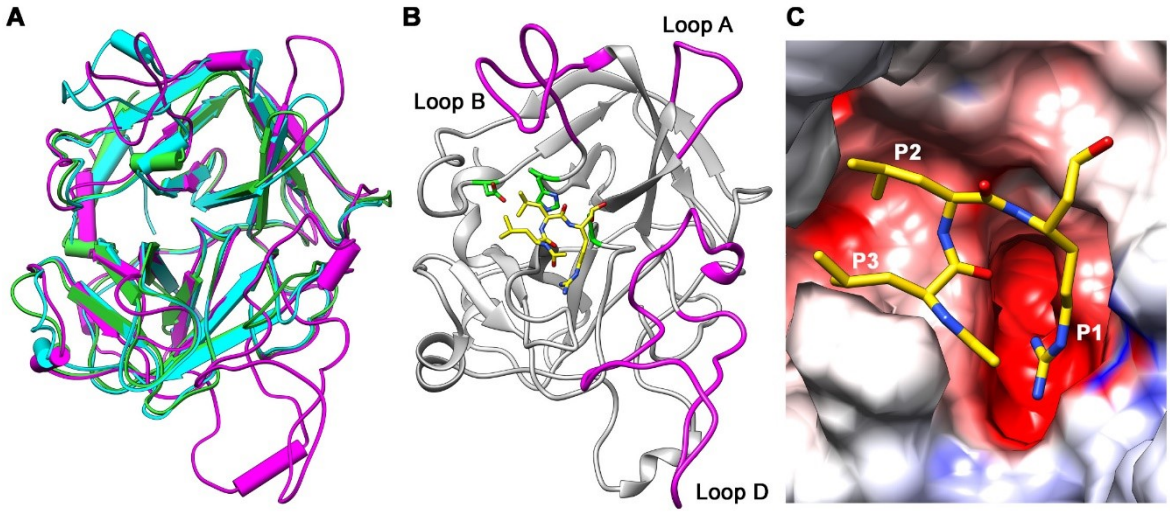

Fig 2. Homology model of the SmSP2 protease domain. The model was built using as a template the X-ray structures of bovine trypsin (PDB 1JRT) and human MASP-1 (PDB 3GOV). (A) A superposition of the SmSP2 model (magenta), the bovine trypsin (green) and MASP-1 (cyan) crystal structures in a cylinder representation. (B) A view from the top on the SmSP2 active site with covalently bound substrate-like inhibitor leupeptin (N-acetyl-L-leucyl-L-leucyl-L-argininal). Carbon atoms of leupeptin are yellow; heteroatoms have the standard color coding (N, blue; O, red). SmSP2 catalytic residues are green. The active site is partially blocked by loops A, B and D (magenta) that are formed by insertions in SmSP2 sequence compared to trypsin sequence. (C) A surface representation of the SmSP2 active site colored by electrostatic potential (at a scale from -10 kT/e (red) to +10 kT/e (blue)). Inhibitor leupeptin is colored as in (B).

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SmSP2 insertions-222 and -251 are located on the top of the loops B and A, respectively (Perona and Craik nomenclature [48]). Their structural analogs can be found in the structure of the MASP-1 catalytic domain where they putatively interact with substrates [49]. The insertion-358 containing 43 residues are located at loop D; however, it has no structural analog in the PDB database (www.rcsb.org) to serve as a template for modeling. The secondary structure prediction did not reveal any conformational element in the loop, suggesting an unstructured character and flexibility that might be involved in interactions during substrate binding.

The ligand binding mode of the SmSP2 protease domain was further analyzed using leupeptin (N-acetyl-L-leucyl-L-leucyl-L-arginal), a transition-state analog protease inhibitor that inhibits SmSP2 (Table 1). Leupeptin was docked into the SmSP2 active site based on the crystallographic complex of this inhibitor with trypsin (PDB code 1JRT). The docking model (Fig 2) suggests that the arginal residue of the inhibitor forms a covalent hemi-acetal linkage with the catalytic Ser447 whereas leupeptin's side chain binds to a deep negatively charged S1 subsite pocket containing Asp441 at the bottom. This type of S1 binding subsite is the primary substrate specificity determinant of trypsin-type proteases and responsible for a substrate/inhibitor preference for basic residues at the P1 position [45].

To conclude, the model of the SmSP2 protease domain indicates that it is a S1 family protease with an trypsin-like substrate binding groove; shielded by surface exposed loops that surround the active site, namely, insertions-222 and -358, may modulate SmSP2's selectivity.

Recombinant expression of SmSP2 and identification of native SmSP2

The protease domain of SmSP2 (rSmSP2, residues 183–501) was expressed in *P. pastoris* and purified. The active enzyme cleaved Z-F-R-AMC and migrated on SDS-PAGE as a single band of approximately 28 kDa (Fig 3A), consistent with the expected molecular mass of 30 kDa.

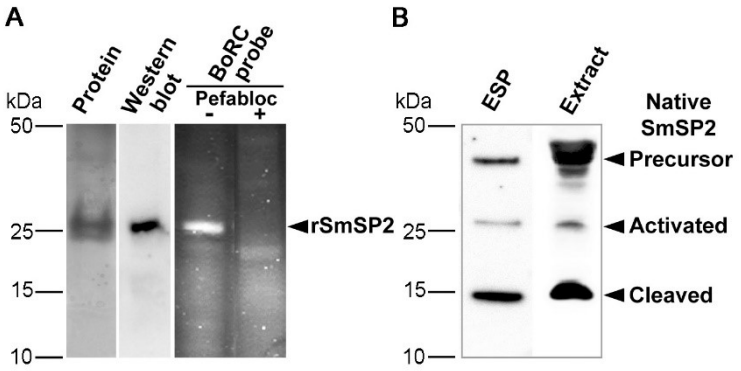

Fig 3. Preparation of recombinant SmSP2 and identification of native SmSP2. (A) The recombinant protease domain of SmSP2 (rSmSP2) expressed in *P. pastoris* was resolved by SDS-PAGE and protein-stained or visualized by polyclonal anti-rSmSP2 IgG. For in-gel activity-based labeling, rSmSP2 was incubated with the fluorescent active site probe, BoRC, resolved by SDS-PAGE and visualized using a fluorescence scanner. The competitive labeling was performed with the serine protease inhibitor, Pefabloc SC. (B) Protein extracts of *S. mansoni* adult worms and their ESP were resolved by SDS-PAGE and visualized by the anti-rSmSP2 IgG.

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rSmSP2 was visualized using the activity-based probe Bodipy-F-P-R-Cmk (BoRC) (Fig 3A) [35]; labeling by the probe was prevented by pre-incubation of the enzyme with the serine protease inhibitor, Pefabloc SC. Rabbit polyclonal antibodies, raised against the SmSP2 protease domain expressed in *E. coli*, reacted with the rSmSP2 by immunoblotting (Fig 3A). In homogenates of adult schistosomes, the antibody recognized three bands of approximately 45, 28 and 15 kDa (Fig 3B), which correspond to the predicted mass of the SmSP2 precursor, the activated SmSP2 protease domain without N-terminal domains and the two-chain form derived by further processing, respectively. Based on its molecular size, we hypothesize that this form was clipped in the insertion-358 loop D. Analogous immunoreactive bands were also demonstrated in the ESP of adult schistosomes (Fig 3B), indicating that SmSP2 is released into the host environment.

The full length SmSP2 sequence contains a stretch of histidine residues that may interact with metal ions. Indeed, native SmSP2 from schistosome extracts bound to a Ni^{2+}- affinity chromatography column and eluted using imidazole in solution (S4 Fig). The eluate contained the three forms of SmSP2 described above. The data also indicate that the activated and clipped protease domains (28 and 15 kDa bands) are disulfide-linked to the N-terminal portion of the molecule that contains the histidine stretch.

Substrate and inhibitor specificity classifies SmSP2 as a trypsin-like enzyme

The substrate specificity of rSmSP2 expressed in *P. pastoris* was initially explored using a panel of specific peptidyl fluorogenic substrates. Two sets of diagnostic protease substrates were used: (i) substrates with a basic amino acid residue (Arg and Lys) at the P1 position that are cleaved by trypsin-like serine proteases, and (ii) substrates containing bulky hydrophobic (Phe and Tyr) or aliphatic residues (Val, Leu and Met) at P1 that are cleaved by chymotrypsin- or elastase-like serine proteases, respectively [50]. The data show that rSmSP2 predominantly hydrolyzed trypsin substrates (Fig 4A). Activity was greatest with Bz-F-V-R-AMC and Z-R-R-AMC, whereas less activity was measured with Z-V-V-R-AMC, Z-V-P-R-AMC and Z-R-R-AMC. The cleavage of related short substrates with free N-termini occurs only very

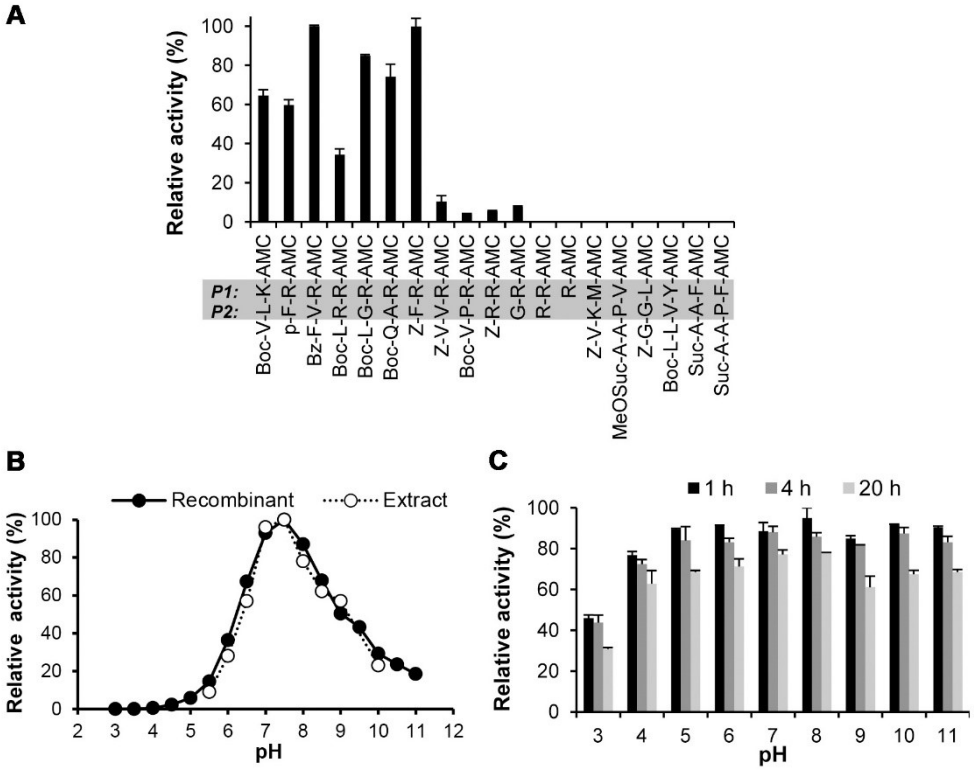

Fig 4. Substrate specificity and pH profile of SmSP2. (A) Activity of rSmSP2 was probed using a panel of peptidyl fluorogenic substrates used to assay trypsin-like and chymotrypsin/elastase-like serine proteases. Substrate hydrolysis was measured in a kinetic assay at pH 8.0. The mean values ± S.D. of three replicates are normalized to the maximum value. Amino acid residues at P1 and P2 positions are highlighted by the grey bar. (B) The pH profiles of rSmSP2 and native SmSP2 activity in extracts of adult worms. Activity was measured in a kinetic assay using the fluorogenic substrate P-F-R-AMC. The native activity (sensitive to the serine protease inhibitor Pefabloc SC) was measured in the presence of 10 μM E-64 and 1 mM EDTA to prevent undesired proteolysis of the substrate by cysteine proteases and metalloproteases, respectively. The mean values of three replicates, expressed as a percentage normalized to the highest value, are shown (standard deviation values are within 5% of the mean). (C) The pH stability of rSmSP2. Activity of rSmSP2 was measured at pH 8.0 in a kinetic assay as in (B) after incubation of the enzyme at pH 3 to 11 for different times. The mean values of three replicates, expressed as a percentage normalized to activity of non-incubated rSmSP2, are shown.

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slowly (G-R-AMC) or not at all (R-R-AMC and R-AMC), suggesting that SmSP2 is not an aminopeptidase trimming N-terminal residues of substrates. The chymotrypsin/elastase substrates were not effectively hydrolyzed.

The pH activity profile of rSmSP2 was determined using the P-F-R-AMC and was similar to that of the serine protease activity in the schistosome adult homogenate (Fig 4B). Effective hydrolysis was observed between pH 6.0 and 10.0 with optimal activity around pH 8.0. No SmSP2 activity was detected below pH 5.0, although rSmSP2 is stable above pH 4.0 (Fig 4C).

To detail the cleavage specificity of rSmSP2, two distinct methods for unbiased substrate profiling were employed. First, a positional scanning-synthetic combinatorial library (PS-SCL) [39] was used to analyze specificity at the substrate positions P1 to P4 (Fig 5A). The cleavage map shows that rSmSP2 prefers basic residues (Lys and Arg) at the P1 position. The P2 and P3 positions display promiscuous specificity, although basic residues at the P2 position, and acidic

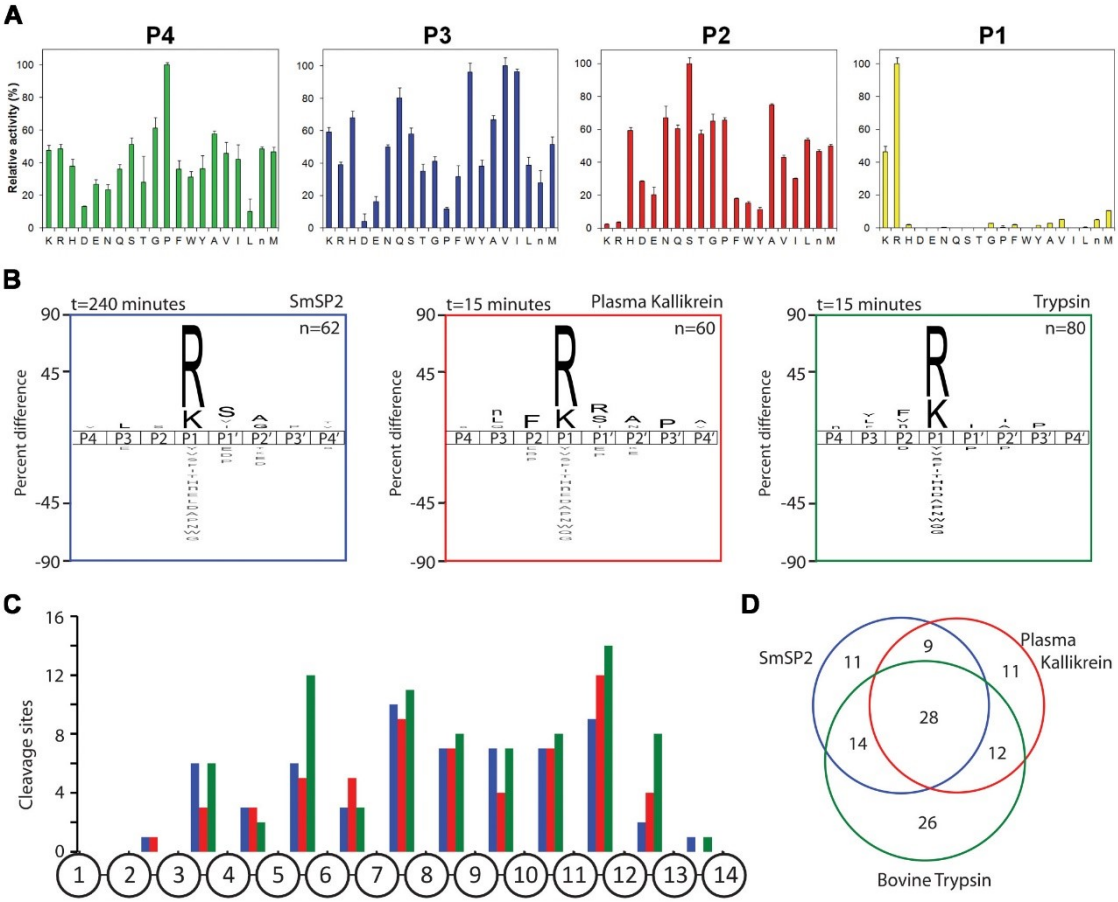

Fig 5. Substrate specificity of rSmSP2. (A) The P1 to P4 specificity of rSmSP2 was determined by a positional scanning-synthetic combinatorial library. The X-axis indicates 20 amino acids held constant at each position (n is norleucine). The Y-axis represents activity related to the most preferred amino acid (100%). (B) The P4 to P4′ specificity profiles of rSmSP2, human plasma kallikrein, and bovine trypsin were obtained using a multiplex combinatorial library (MSP-MS). The iceLogo substrate profiles were generated from the pattern of cleavage events after incubation with the 14-mer library. Amino acids that are most frequently found at each position are shown above the horizontal line, whereas amino acids that less frequently observed are below. (C) Spatial distribution of cleavage sites within the 14-mer peptide scaffold. (D) The Venn diagram shows the number of unique and shared cleavage sites.

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residues and Pro at the P3 position are unfavorable. Some degree of selectivity was measured for Pro at P4. Second, a sensitive multiplex substrate profiling by mass spectrometry (MSP-MS) was performed with a library of 228 tetradecapeptides [51] to analyze the subsites on both sides of the scissile peptide bond (Fig 5B). The MSP-MS profile confirmed the strong preference for Arg and Lys residues at the P1 position. At the primed sites, the preferred residues were Ser at the P1′, Ala and Gly at P2′ positions, respectively. Compared to human plasma kallikrein and bovine trypsin, the MSP-MS cleavage profile of SmSP2 were similar (Fig 5D) with 28 identical cleavage sites. However, trypsin produced 26 unique cleavages compared to 11 cleavages unique for rSmSP2 or plasma kallikrein. In general, cleaved peptide bonds

occurred away from the amino and carboxyl termini confirming endopeptidase mode of action of all three analyzed enzymes (Fig 5C).

The inhibitor specificity of rSmSP2 was investigated using a panel of small molecule and protein inhibitors (Table 1). rSmSP2 was highly sensitive to inhibitors of S1 family serine proteases, including the small-molecules, Pefabloc SC, benzamidine, leupeptin, antipain, and 3,4-dichlorcoumarin, and the proteinaceous soybean trypsin (STI) and bovine pancreatic trypsin inhibitors (BPTI). rSmSP2 was not affected by inhibitors of aspartic, cysteine and metalloproteases. Protein inhibitors of the serpin family inhibited rSmSP2 with variable efficiencies: PAI-I, α-1-antitrypsin and anti-thrombin III, which interact with trypsin-like proteases, inhibited rSmSP2 activity strongly, whereas the chymotrypsin protease inhibitor, α-1-antichymotrypsin, was weakly effective.

To summarize, SmSP2 hydrolyzes substrates as an endopeptidase and has a strict specificity for basic residues at P1.

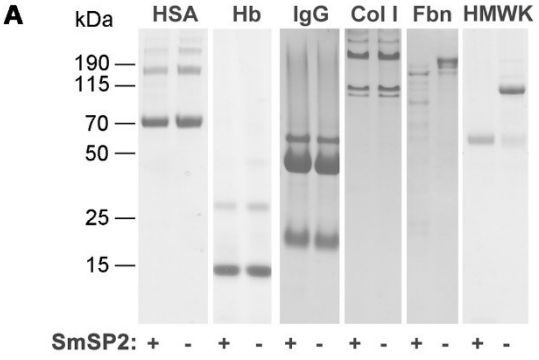
SmSP2 releases bradykinin from kininogen and activates plasmin

The activity of rSmSP2 towards host-derived macromolecular substrates was tested with several human and bovine proteins. After incubation, the mixtures were subsequently analyzed by SDS-PAGE (Fig 6A). No hydrolysis was observed for hemoglobin and serum albumin, the two major protein components of vertebrate host blood. Also, neither immunoglobulin G nor collagen was cleaved by rSmSP2. On the other hand, rSmSP2 completely hydrolyzed the blood clotting protein, fibronectin. We also analyzed the processing of three blood proteins: HMWK, tPA and human plasminogen.

Amino acid sequencing showed that HMWK was processed to the kininogen light chain (Fig 6A), and that the heavy chain was completely fragmented. LC-MS/MS analysis of the reaction mixture revealed the release of the HMWK-derived vasodilatory nonapeptide, bradykinin (Fig 6B). To simulate the bradykinin release from HMWK precursor, we designed a synthetic hexadecapeptide (Ac-SLMKRPPGFSPFRSSR-amide) designated extended bradykinin. This peptide was derived from the HMWK sequence to contain processing sites; *i.e.*, the bradykinin sequence (RPPGFSPFR) was extended at the N- terminus by four additional HMWK residues (SLMK) and three residues on C-terminus (SSR). After incubation of this peptide with rSmSP2, the resulting fragments were separated by HPLC and the cleavage positions identified by mass spectrometry. The precursor was cleaved between Lys-Arg and Arg-Ser bonds thereby releasing bradykinin (Fig 6C). Synthetic bradykinin or kallidin (lysyl-bradykinin) were not cleaved by rSmSP2. Also, rSmSP2 degraded vasopressin, a nonapeptide hormone that increases arterial blood pressure by inducing vasoconstriction.

Next, we investigated whether living schistosomes produce a cleavage specificity similar to that of SmSP2 when incubating with vasopressin or extended bradykinin in culture. Adult schistosomes were incubated in the presence of the peptides and cleavage positions analyzed by mass spectrometry (Fig 6C). Both peptides were cleaved when added to the cultivation medium: vasopressin was inactivated by cleavage after penultimate Arg residue; extended bradykinin was cleaved to release bradykinin, however, cleavage precursors extended in N- or C-terminal direction were also identified. The fragmentation was significantly abolished in the presence of a serine protease specific inhibitor Pefabloc. The identified cleavage positions in the hormone sequences were identical with those obtained by *in vitro* fragmentation using rSmSP2.

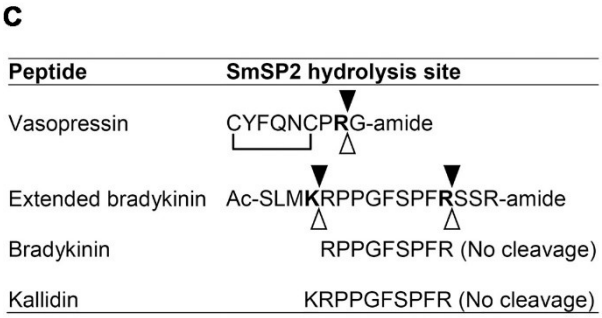
The processing of human plasminogen, the precursor of the main fibrinolytic protease plasmin, by rSmSP2 was analyzed by SDS-PAGE and kinetic assay. Single chain plasminogen was completely converted into plasmin, consisting of a heavy chain and light chain). This

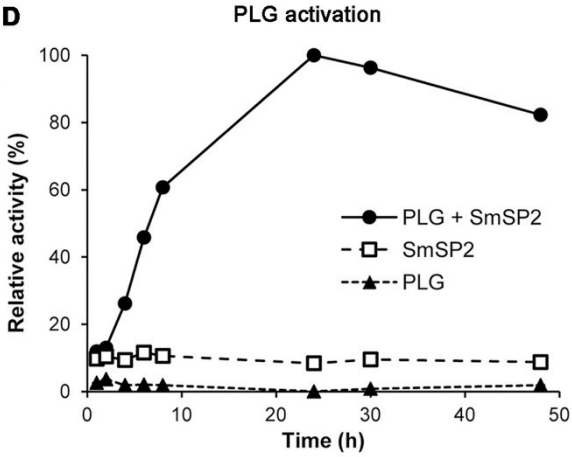


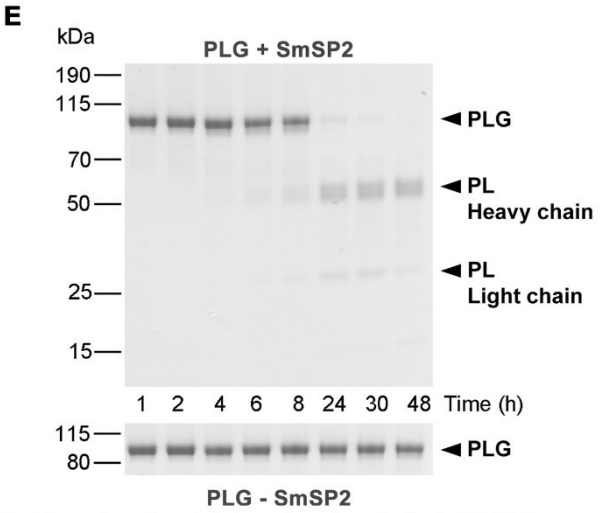
B

Bradykinin release from HMWK

Bradykinin sequence	Theoretical mass [M] (Da)	Measured mass [M] (Da)	Delta (ppm)
RPPGFSPFR	1059.561	1059.564	2.83







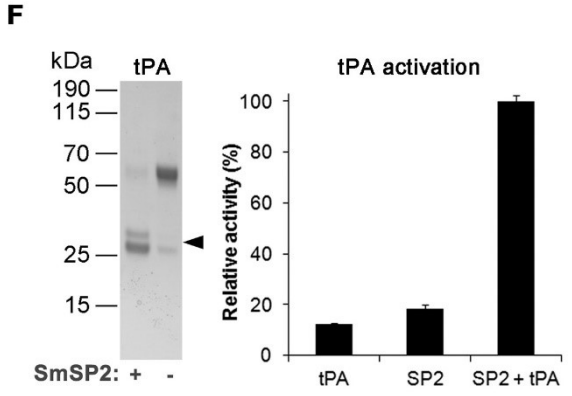


Fig 6. Processing of host-derived proteins and peptides by rSmSP2. (A) Human serum albumin (HSA), hemoglobin (Hb), immunoglobulin G (IgG), collagen type I (Col I), fibronectin (Fbn) and high molecular weight kininogen (HMWK) were incubated for 16 h at pH 8.0 in the presence (+) or absence (-) of rSmSP2. The reaction mixtures were subjected to SDS-PAGE and protein stained. (B) HMWK was incubated with rSmSP2 and the reaction mixture was subjected to LC-MS/MS analysis to identify bradykinin peptide released from HMWK. (C) Peptide hormones were incubated with rSmSP2 or with live adults maintained in culture and the cleavage positions (full triangles for rSmSP2, open triangles for adult schistosomes) were identified by mass spectrometry. Residues at the P1 position are in bold and the disulfide

connectivity of vasopressin is indicated. (D) Human plasminogen (PLG) was incubated in the presence or absence of rSmSP2 and the reaction mixture was analyzed at different time points. Plasmin proteolytic activity generated during plasminogen processing by rSmSP2 was determined in a kinetic assay with Boc-V-L-K-AMC. Mean values of triplicates are expressed relative to the maximum value (100%). The S.D. values of three replicates are within 10% of the mean. All experiments were performed at least twice with similar results. (E) The processed forms were resolved by SDS-PAGE and visualized by protein staining. The positions for PLG, and plasmin (PL) heavy and light chains are indicated. (F) Human tissue plasminogen activator (tPA) was incubated for 16 h at pH 8.0 in the presence/absence of rSmSP2 and analyzed by SDS-PAGE with protein staining; proteolytic activity generated during tPA processing was monitored in a kinetic assay using Z-G-G-R-AMC. Mean values ± s.d. of triplicates are expressed relative to the maximum value (100%). Two chain tPA is indicated with an arrow.

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processing was not the result of the self-activation as it only occurred in the presence of rSMSP2 (Fig 6D and 6E). SDS-PAGE analysis showed that SmSP2 processes single-chain tPA into its two-chain form; the cleavage was associated with a ten-fold increase in tPA activity (Fig 6F). tPA is the major physiological activator of plasminogen, thus the SmSP2 cleavage of single chain tPA into its fully active two-chain form would result in more efficient plasmin activation and in turn more effective fibrinolysis.

To conclude, SmSP2 cleaves several physiologically important blood proteins. Specifically, it processes the extracellular matrix and blood clot component, fibronectin, activates the major fibrinolytic enzyme, plasmin, and its activator, tPA, releases the vasodilatory peptide bradykinin from its HMWK precursor and processes the vasoconstrictory peptide vasopressin.

SmSP2 is localized in the tegument, parenchyma and reproductive organs of adult schistosomes

Indirect immunofluorescence microscopy on semi-thin sections using affinity purified polyclonal antibodies raised against rSmSP2 demonstrated that SmSP2 is expressed in distinct tissues of adult worms (Fig 7 and S5 Fig). In males, SmSP2 was observed in the parenchyma, tegument and in the tegumental surface membranes except the tubercles (for a detailed tegumental localization, see S5 Fig). In females, SmSP2 was observed in parenchyma but not in the tegument. In addition, intense staining was seen in the esophageal region of both genders, in the testes of males and in the ovaria and vitellaria of females (Fig 7). SmSP2 was absent from muscle, the tegumental tubercles, gastrodermis and gut lumen. Pre-immune serum was used as a negative control (S6 Fig) and only faint background fluorescence was detected.

Discussion

Serine proteases of the S1 family are key factors of virulence for many parasitic helminths. They contribute to parasite invasion, migration, nutrition and reproduction, and facilitate adaption to and evasion of the host's physiological and immune responses (for reviews see [12, 52]). Among serine proteases, most attention has been focused on *S. mansoni* cercarial elastases as these enzymes are implicated in tissue invasion and migration into the definitive mammalian hosts [13, 53]; however, information regarding other SmSPs is limited. Recently, we described the repertoire of non-cercarial elastase SmSPs by employing a series of genomic, transcriptomic, functional proteomic and phylogenetic approaches [23].

Here, we focus on SmSP2 as it is the most abundantly expressed SP in the *Schistosoma* blood-dwelling developmental stages [23]. The domain organization of SmSP2 is distantly reminiscent of the modular architecture of host blood-clotting serine proteases. Specifically, these enzymes contain a catalytic trypsin-like serine protease domain linked by disulfide bonds to an N-terminal multi-domain region that is involved in ligand-binding and protein-protein interactions [54]. SmSP2 also has unique features—an N-terminal region histidine stretch and a TSR-1 domain. TSR-1 domains mediate cell adhesion, protein-protein interactions and glycosaminoglycans binding [42, 43]. The histidine stretch may act as a metal binding site for divalent metal ions, as we demonstrated *in vitro* using metal-affinity

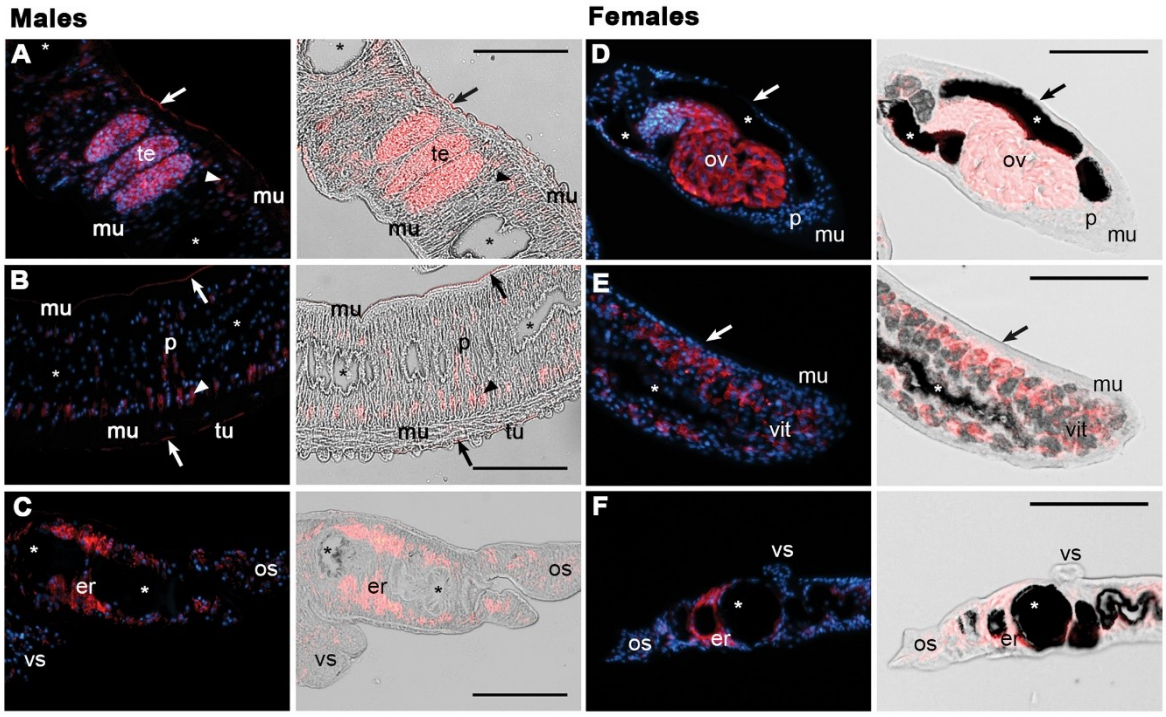

Fig 7. SmSP2 immunolocalization in sections of adult *S. mansoni*. Semi-thin sections of adult *S. mansoni* males (A to C) and females (D to F) were probed with an anti-SmSP2 IgG followed by reaction with an anti-rabbit Alexa 647-labeled secondary antibody (red). DAPI was used to label nuclear DNA (blue). The left columns show merged fluorescent channels; in the right columns, the signal is merged with differential interference contrast. A strong SmSP2 signal (red) was detected in both sexes in the parenchyma (p) and the esophageal region (er). A faint signal was noted in the ventral (vs) and oral suckers (os). No signal was detected in the gut (asterisks), muscle (mu) or tegumental tubercles (tu). In males, SmSP2 signal also appears in the tegument (arrowhead) in the tegumental membrane surface (arrow) and in the testes (te). In females, the signal is noted in the ovaries (ov) and vitellaria (vit). The scale bar represents 100 μm. A and D, reproductive organs; B and E, tegumental cells; C and F, head.

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chromatography to purify native SmSP2 from the adult schistosome homogenate. The TSR-1 domain is also present in the sequences of SmSP2 orthologs in other trematodes; cestodes retain only the TSR-1 domain while the histidine stretch is unique to trematodes (S3 Fig). Orthologous genes were not found in other organisms. Further research is needed to evaluate the exact function of the N-terminal domains of SmSP2.

SmSP2 orthologs (termed mastins) are present in S. *haematobium* and *S. japonicum*. For *S. haematobium* infections, mastin was identified in a protein array as an antigen targeted by a protective IgG1 immune response in those individuals with acquired resistance and, therefore, suggested as an attractive vaccine candidate [24]. This resistance is acquired after treatment with PZQ and the consequent exposure of parasite antigens to the host immune system [55]. SmSP2 orthologs are also present in the larval stage (cysticercus) of the cestodes, *Echinococcus granulosus* [56] and *Taenia solium* [57], and were identified as Antigen 5 excretory proteins (Ag5). Ag5 proteins are major components of cyst fluid and are used in a serodiagnostic test for cysticercosis [57, 58]. In comparison with SmSP2, the catalytic serine residue is replaced by threonine and Ag5 proteins show only marginal proteolytic activity [56, 57].

In adult *S. mansoni*, we demonstrate that SmSP2 is localized in the tegument. Treatment with PZQ causes tegumental damage [59, 60] and thereby exposes schistosome antigens, including SmSP2, to the host immune system [55]. The tegumental location may explain the recognition of SmSP2 by sera of PZQ-treated individuals [24]. Also, the localization of SmSP2 to the esophagus may indicate that the enzyme facilitates some aspect of nutrition during the ingestion of host proteins or is secreted. Indeed, we observed that SmSP2 is found in the ESP as was noted in *E. granulosus* previously for Ag5 [58, 61]. In addition, SmSP2 and its *S. japonicum* mastin ortholog were recently proteomically identified in exosome-like vesicles that are secreted by parasite and putatively modulate host-parasite interactions [62–64]. Finally, apart from a potential extracorporeal function, the protease domain of SmSP2 was localized to a number of internal tissues, including the ovaries, testes, muscle and parenchyma, suggesting a variety of functional roles.

To enzymatically characterize SmSP2, the protease domain was heterologously expressed in *P. pastoris* and purified as an active protease. SmSP2 was subjected to a range of biochemical analyses to determine its substrate and inhibitory specificity. SmSP2 was classified as a trypsin-like enzyme as it cleaves various peptide substrates in an endopeptidolytic mode at the carboxyl terminus of Arg or Lys residues and was inhibited by inhibitors targeting trypsin-like proteases such as leupeptin, benzamidine and antipain [65]. Consistent with this, systematic cleavage specificity analysis with the positional-scanning and multiplex substrate libraries revealed a preference for basic amino acids at P1. In agreement with the cleavage specificities, the homology model of SmSP2 reveals that the S1 binding pocket has an architecture analogous to vertebrate trypsins, including the critical Asp441 residue that defines the preference for basic P1 residues [45].

Based on homology modeling, the SmSP2 protease domain contains a large 43 residue-long insertion (at position 358) which is a unique structural feature of SmSP2 and its trematode orthologs not present in other S1 family proteases. This insertion is localized in the vicinity of the active site on the loop D (nomenclature according to Perona and Craik [48]). We demonstrate that SmSP2 performs limited proteolysis to process a number of physiologically- relevant host proteins: it degrades fibronectin, activates plasmin and tPA and releases bradykinin from its precursor HMWK. SmSP2 is incapable of cleaving macromolecular substrates such as Hb, BSA and IgG, which, for example, are cleaved by the gut-associated cysteine and aspartic proteases [15, 16]. SmSP2 has a more narrow substrate specificity than trypsin, the S1 family archetypal protease. In multiplex cleavage assays, trypsin cleaved more substrates than SmSP2. It resulted in similar number of cleavages produced by trypsin in 15 min whereas 4 h was required by SmSP2. A plausible explanation is that the B and D loop-insertions on SmSP2 limit the access of substrates to the active site resulting in the selective processing of conformationally compatible peptides. However, the insertion-D does not directly occlude the active site subsites as this would confer an exopeptidase activity that is not observed for SmSP2.

Adult schistosomes can survive for decades in the host [66]. It is thought that these large intravascular parasites manipulate the complex hemostatic system of the host at different levels via bioactive molecules in the ESP or on the tegument [67]. However, the detailed molecular processes underlying these survival mechanisms are not fully understood. Our work demonstrates that SmSP2 is present in both the ESP and tegument. We show that SmSP2 possesses a kallikrein-like activity as it cleaves the plasma protein kininogen to generate the peptide hormone, bradykinin. Bradykinin is a potent vasodilator that decreases blood pressure and increases vascular permeability [68]. Bradykinin also exerts anti-thrombogenic, anti-proliferative and anti-fibrogenic effects [69]. In a recent report, we demonstrated that living schistosomes cleave bradykinin and angiotensin I (converting this vasoconstrictor to the vasodilatory angiotensin-(1–7)), and that the tegumental, S9 serine protease, SmPOP, is involved in that

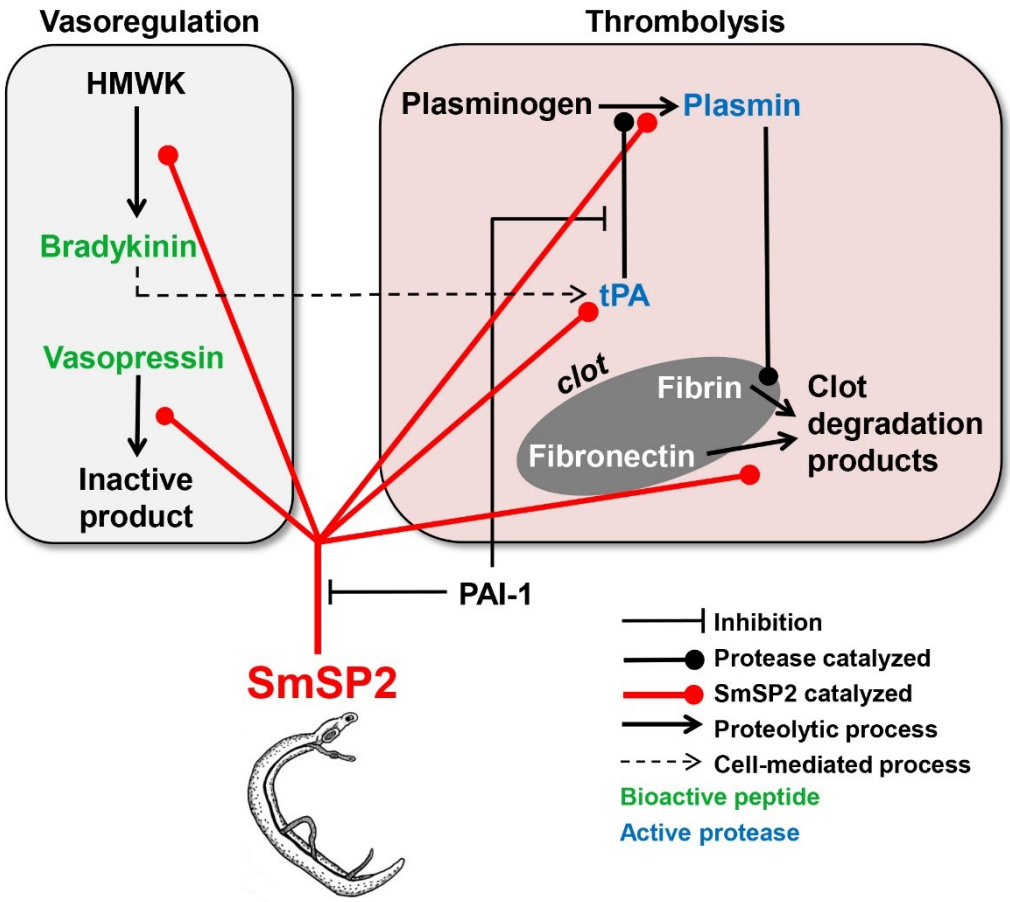

Fig 8. Model of how SmSP2 may interfere with the host's hemostatic system. SmSP2, secreted from adult schistosomes or localized at the surface, stimulates the degradation of blood clots (thrombolysis panel) by (i) activation of two critical components of the fibrinolytic system, tissue plasminogen activator (tPA) and plasminogen, and (ii) direct degradation of the blood-clot component, fibronectin. SmSP2 modulates vascular tone (vasoregulation panel) by processing bioactive peptide hormones. (i) It releases the vasodilatory bradykinin from kininogen (HMWK) and (ii) degrades the vasoconstrictory peptide, vasopressin. Bradykinin may stimulate the release of tPA from vascular endothelial cells (dashed line) which would promote fibrinolysis. SmSP2 may be regulated by plasminogen activator inhibitor-1 (PAI-1) that inhibits SmSP2 (Table 1).

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processing [37]. Moreover, SmSP2 inactivates vasopressin, a hormone that increases arterial blood pressure by inducing vasoconstriction. It is, therefore, possible that both SmPOP and SmSP2 modulate the parasite's local vascular environment to the parasite's benefit during its residence and movement in the host's blood vessels. Additionally, we show that the whole schistosome parasite can cleave the vasopressin and release bradykinin from its synthetic precursor, when co-incubated *in vitro*. The activity is due to a serine protease(s) (possibly SmSP2) that is most abundantly expressed serine protease in adult schistosome [23] as indicated by mRNA expression levels, mass spectrometry and specific inhibition by a serine protease inhibitor.

Due to their large size, adult schistosomes may alter or disrupt normal blood flow, and damage the endothelium, which could lead to platelet activation and subsequently blood coagulation [67]. However, blood clots are not observed around the parasites residing in the host blood vessels and several mechanisms have been proposed with which schistosomes inhibit blood clot formation and/or promote blood clot lysis [70]. Physiologically, the latter process involves proteolytic degradation of fibrin that is mediated by plasmin. This central protease in the fibrinolytic system is generated from its zymogen, plasminogen (PLG), by, for example, tissue plasminogen activator (tPA). Based on our *in vitro* results, we propose here a new and complex mechanism with which schistosomes employ SmSP2 to promote fibrinolysis at multiple levels (Fig 8): 1) SmSP2 activates PLG to plasmin; this action can be accelerated by presentation of PLG on tegumental receptors (*e.g.*, enolase [71] of schistosomes); 2) SmSP2 processes the single-chain tPA to the more potent double-chain tPA form [72] which would then cause enhanced plasmin activation, 3) SmSP2 produces bradykinin that is known to stimulate the release of tPA from the vascular endothelium [73]; and 4) SmSP2 directly degrades the blood clot component fibronectin, which has also been recently shown for a tegumental calpain [74].

To conclude, we have expressed, and biochemical and functionally characterized the multidomain serine protease, SmSP2. The protease is a putative anti-hemostatic peculiar to platyhelminths, which are the only pathogens to express SmSP2 orthologs. Further research is needed to evaluate the role of SmSP2 (and orthologs) in modulating host-parasite interactions and its potential as a drug or vaccine candidate.

Supporting information

S1 Fig. Multiple sequence alignment of the TSR-1 domain of SmSP2 with selected TSR-1 domains of human proteins. Sequences are: TSP-1-1-3—thrombospondin-1 (TSP) type-1 domains 1, 2 and 3 (Uniprot accession number: P07996), properdin-TSR1-6—properdin thrombospondin type-1 domains 1–6 (P27918), ADAMTS13 (Q76LX8), and spondin-TSP-1—spondin-1 thrombospondin type-1 domain 1 (Q9HCB6). Cys residues are highlighted in yellow, tryptophan substituents forming the W layer are in blue, and amino acid residues forming the R layers are in green.
(TIF)

S2 Fig. Multiple sequence alignment of the SmSP2 protease domain with catalytic domains of selected human and bovine S1 family proteases. Human proteases: mannan-binding lectin serine protease 1 (MASP-1, Uniprot accession number: P48740), tissue plasminogen activator (tPA, P00750), urokinase plasminogen activator (uPA, P00749), plasmin (P00747), kallikrein 1 (P06870) and matriptase (MTSP-1, Q9Y5Y6). Bovine proteases: cationic trypsin (P00760) and chymotrypsin A (P00766). The catalytic triad residues (His, Asp, Ser) are red-boxed; the critical Asp residue in the S1 subsite that accounts for trypsin-like activity is green-boxed. Cys residues that are predicted to form disulfide bonds are indicated by the same color, cyan Cys form interchain disulfide bond with domains not included in the alignment. Residues that are shared between sequences are shaded in grey. Residues forming SmSP2 insertion-222, 251, and 358 are underlined. The upper line numbering is according to SmSP2, the lower line numbering according to bovine chymotrypsinogen.
(TIF)

S3 Fig. Multiple sequence alignment of SmSP2 with orthologs from other platyhelminth parasites. Trematode sequences: *Schistosoma. japonicum* (GenBank: AAW24683.1), *Schistosoma haematobium* (XP_012796372.1), *Fasciola hepatica* (sequence identified in the transcriptome database (Young et al. (2010), Biotechnol Adv 28, 222–231), *Opisthorchis viverrini*

(XP_009167273.1) and *Clonorchis sinensis* (GAA32831.2). Cestode sequences: *Hymenolepis microstoma* (CDS25513.1), *Echinococcus multiocularis* (CDI97096.1), *Echinococcus granulosus* (EUB58856.1) and *Taenia solium* (ADP89566.1). Predicted signal sequences are in blue, histidine residues in the N-terminal region are in purple and the TSR-1 domain is in green. The catalytic triad residues (His, Asp, Ser) are red-boxed, the critical Asp residue in S1 subsite that accounts for trypsin-like activity is green-boxed. Cys residues in the TSR-1 domain are highlighted in yellow and Cys residues in the protease domain are in cyan.
(TIF)

S4 Fig. Binding of native SmSP2 to a Ni^{2+}-ion affinity column. A protein extract of adult schistosomes (Extract) was applied to a HiTrap IMAC FF column containing immobilized Ni^{2+} ions and native SmSP2 eluted using 0.5 M imidazole. The extract, unbound material (FT) and eluted material (Elution) were resolved by SDS-PAGE, electrophoretically transferred onto a PVDF membrane and visualized by anti-rSmSP2 IgG.
(TIF)

S5 Fig. Detailed micrograph of SmSP2 localization in the tegument of adult male *S. mansoni*. The tissue section was probed with anti-SmSP2 IgG followed by an anti-rabbit IgG Alexa 594-labeled secondary antibody (red). DAPI was used to label the nuclear DNA (blue). The left image shows merged fluorescent channels; on the right, schematic depiction of the adult schistosome surface; sm—surface membrane, tg—tegument, mu—muscle, tc—tegumental cell (cyton), pa—parenchym.
(TIF)

S6 Fig. Pre-immune serum is not reactive. As a negative control, semi-thin sections of adult S. mansoni males and females were probed with a pre-immune serum (A-F) followed by reaction with an anti-rabbit IgG Alexa 647-labeled secondary antibody (red). DAPI was used to label nuclear DNA (blue). The first and third columns show merged fluorescent channels; in the second and fourth columns, the signal is merged with differential interference contrast.
(TIF)

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Writing – original draft: Adrian Leontovyč.

Writing – review & editing: Anthony J. O'Donoghue, Marta Chanová, Charles S. Craik, Conor R. Caffrey, Michael Mareš, Jan Dvořák, Martin Horn.

References

1. Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect Dis. 2006; 6 (7):411–25. S1473-3099(06)70521-7 [pii]; https://doi.org/10.1016/S1473-3099(06)70521-7 PMID: 16790382
2. King CH. Parasites and poverty: the case of schistosomiasis. Acta Trop. 2010; 113(2):95–104. https://doi.org/10.1016/j.actatropica.2009.11.012 PMID: 19962954; PubMed Central PMCID: PMCPMC2812649.
3. Gryseels B, Polman K, Clerinx J, Kestens L. Human schistosomiasis. Lancet. 2006; 368(9541):1106–18. https://doi.org/10.1016/S0140-6736(06)69440-3 PMID: 16997665
4. Burke ML, Jones MK, Gobert GN, Li YS, Ellis MK, McManus DP. Immunopathogenesis of human schistosomiasis. Parasite Immunol. 2009; 31(4):163–76. PIM1098 [pii]; https://doi.org/10.1111/j.1365-3024.2009.01098.x PMID: 19292768
5. Caffrey CR. Chemotherapy of schistosomiasis: present and future. Curr Opin Chem Biol. 2007; 11 (4):433–9. https://doi.org/10.1016/j.cbpa.2007.05.031 PMID: 17652008
6. Caffrey CR. Schistosomiasis and its treatment. Future Med Chem. 2015; 7(6):675–6. https://doi.org/10.4155/fmc.15.27 PMID: 25996057.
7. Skelly PJ, Alan WR. Making sense of the schistosome surface. Adv Parasitol. 2006; 63:185–284. S0065-308X(06)63003-0 [pii]; https://doi.org/10.1016/S0065-308X(06)63003-0 PMID: 17134654
8. Da'dara A, Skelly PJ. Manipulation of vascular function by blood flukes? Blood Rev. 2011; 25(4):175–9. S0268-960X(11)00034-8 [pii]; https://doi.org/10.1016/j.blre.2011.04.002 PMID: 21543145
9. de Oliveira Fraga LA, Lamb EW, Moreno EC, Chatterjee M, Dvorak J, Delcroix M, et al. Rapid induction of IgE responses to a worm cysteine protease during murine pre-patent schistosome infection. BMC Immunol. 2010; 11:56. 1471-2172-11-56 [pii]; https://doi.org/10.1186/1471-2172-11-56 PMID: 21078176
10. Schramm G, Falcone FH, Gronow A, Haisch K, Mamat U, Doenhoff MJ, et al. Molecular characterization of an interleukin-4-inducing factor from Schistosoma mansoni eggs. J Biol Chem. 2003; 278 (20):18384–92. https://doi.org/10.1074/jbc.M300497200 PMID: 12624091.
11. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, Cerqueira GC, et al. The genome of the blood fluke Schistosoma mansoni. Nature. 2009; 460(7253):352–8. https://doi.org/10.1038/nature08160 PMID: 19606141
12. McKerrow JH, Caffrey C, Kelly B, Loke P, Sajid M. Proteases in parasitic diseases. Annu Rev Pathol. 2006; 1:497–536. https://doi.org/10.1146/annurev.pathol.1.110304.100151 PMID: 18039124
13. Salter JP, Choe Y, Albrecht H, Franklin C, Lim KC, Craik CS, et al. Cercarial elastase is encoded by a functionally conserved gene family across multiple species of schistosomes. J Biol Chem. 2002; 277 (27):24618–24. https://doi.org/10.1074/jbc.M202364200 PMID: 11986325.
14. Lim KC, Sun E, Bahgat M, Bucks D, Guy R, Hinz RS, et al. Blockage of skin invasion by schistosome cercariae by serine protease inhibitors. American Journal of Tropical Medicine and Hygiene. 1999; 60 (3):487–92. WOS:000079506900026. PMID: 10466982
15. Delcroix M, Sajid M, Caffrey CR, Lim KC, Dvorak J, Hsieh I, et al. A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. J Biol Chem. 2006; 281(51):39316–29. https://doi.org/10.1074/jbc.M607128200 PMID: 17028179
16. Caffrey CR, McKerrow JH, Salter JP, Sajid M. Blood 'n' guts: an update on schistosome digestive peptidases. Trends Parasitol. 2004; 20(5):241–8. https://doi.org/10.1016/j.pt.2004.03.004 PMID: 15105025
17. Abdulla MH, Lim KC, Sajid M, McKerrow JH, Caffrey CR. Schistosomiasis mansoni: novel chemotherapy using a cysteine protease inhibitor. PLoS Med. 2007; 4(1):e14. https://doi.org/10.1371/journal.pmed.0040014 PMID: 17214506

18. Fanfrlik J, Brahmkshatriya PS, Rezac J, Jilkova A, Horn M, Mares M, et al. Quantum Mechanics-Based Scoring Rationalizes the Irreversible Inactivation of Parasitic Schistosoma mansoni Cysteine Peptidase by Vinyl Sulfone Inhibitors. Journal of Physical Chemistry B. 2013; 117(48):14973–82. https://doi.org/10.1021/jp409604n WOS:000328101100010. PMID: 24195769

19. Horn M, Jilkova A, Vondrasek J, Maresova L, Caffrey CR, Mares M. Mapping the Pro-Peptide of the Schistosoma mansoni Cathepsin B1 Drug Target: Modulation of Inhibition by Heparin and Design of Mimetic Inhibitors. Acs Chemical Biology. 2011; 6(6):609–17. https://doi.org/10.1021/cb100411v WOS:000291896400011. PMID: 21375333

20. Jilkova A, Rezacova P, Lepsik M, Horn M, Vachova J, Fanfrlik J, et al. Structural Basis for Inhibition of Cathepsin B Drug Target from the Human Blood Fluke, Schistosoma mansoni. Journal of Biological Chemistry. 2011; 286(41):35770–81. https://doi.org/10.1074/jbc.M111.271304 WOS:000295927100050. PMID: 21832058

21. Ingram JR, Rafi SB, Eroy-Reveles AA, Ray M, Lambeth L, Hsieh I, et al. Investigation of the proteolytic functions of an expanded cercarial elastase gene family in Schistosoma mansoni. PLoS Negl Trop Dis. 2012; 6(4):e1589. https://doi.org/10.1371/journal.pntd.0001589 PNTD-D-11-01296 [pii]. PMID: 22509414

22. Dvorak J, Horn M. Serine proteases in schistosomes and other trematodes. Int J Parasitol. 2018; 48 (5):333–344. https://doi.org/10.1016/j.ijpara.2018.01.001 PMID: 29477711.

23. Horn M, Fajtova P, Arreola LR, Ulrychova L, Bartosova-Sojkova P, Franta Z, et al. Trypsin- and Chymotrypsin-Like Serine Proteases in Schistosoma mansoni—'The Undiscovered Country'. Plos Neglected Tropical Diseases. 2014; 8(3). https://doi.org/10.1371/journal.pntd.0002766 WOS:000337348800041. PMID: 24676141

24. Pearson MS, Becker L, Driguez P, Young ND, Gaze S, Mendes T, et al. Of monkeys and men: immunomic profiling of sera from humans and non-human primates resistant to schistosomiasis reveals novel potential vaccine candidates. Frontiers in immunology. 2015; 6:213. Epub 2015/05/23. https://doi.org/10.3389/fimmu.2015.00213 PMID: 25999951; PubMed Central PMCID: PMC4419842.

25. Dvorak J, Mashiyama ST, Sajid M, Braschi S, Delcroix M, Schneider EL, et al. SmCL3, a gastrodermal cysteine protease of the human blood fluke Schistosoma mansoni. PLoS Negl Trop Dis. 2009; 3(6): e449. https://doi.org/10.1371/journal.pntd.0000449 PMID: 19488406

26. Stefanic S, Dvorak J, Horn M, Braschi S, Sojka D, Ruelas DS, et al. RNA Interference in Schistosoma mansoni Schistosomula: Selectivity, Sensitivity and Operation for Larger-Scale Screening. Plos Neglected Tropical Diseases. 2010; 4(10). https://doi.org/10.1371/journal.pntd.0000850 WOS:000283559600019. PMID: 20976050

27. Duvall RH, DeWitt WB. An improved perfusion technique for recovering adult schistosomes from laboratory animals. Am J Trop Med Hyg. 1967; 16(4):483–6. PMID: 4952149.

28. Basch PF. Cultivation of Schistosoma mansoni in vitro. I. Establishment of cultures from cercariae and development until pairing. J Parasitol. 1981; 67(2):179–85. PMID: 7241277

29. Dvorak J, Fajtova P, Ulrychova L, Leontovyc A, Rojo-Arreola L, Suzuki BM, et al. Excretion/secretion products from Schistosoma mansoni adults, eggs and schistosomula have unique peptidase specificity profiles. Biochimie. 2016; 122:99–109. https://doi.org/10.1016/j.biochi.2015.09.025 WOS:000370910700010. PMID: 26409899

30. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011; 8(10):785–6. https://doi.org/10.1038/nmeth.1701 PMID: 21959131.

31. Caffrey CR, Salter JP, Lucas KD, Khiem D, Hsieh I, Lim KC, et al. SmCB2, a novel tegumental cathepsin B from adult Schistosoma mansoni. Mol Biochem Parasitol. 2002; 121(1):49–61. PMID: 11985862

32. Jilkova A, Horn M, Rezacova P, Maresova L, Fajtova P, Brynda J, et al. Activation Route of the Schistosoma mansoni Cathepsin B1 Drug Target: Structural Map with a Glycosaminoglycan Switch. Structure. 2014; 22(12):1786–98. https://doi.org/10.1016/j.str.2014.09.015 WOS:000345898700012. PMID: 25456815

33. Sojka D, Franta Z, Frantova H, Bartosova P, Horn M, Vachova J, et al. Characterization of Gut-associated Cathepsin D Hemoglobinase from Tick Ixodes ricinus (IrCD1). Journal of Biological Chemistry. 2012; 287(25):21152–63. https://doi.org/10.1074/jbc.M112.347922 WOS:000306416800036. PMID: 22539347

34. Sajid M, McKerrow JH, Hansell E, Mathieu MA, Lucas KD, Hsieh I, et al. Functional expression and characterization of Schistosoma mansoni cathepsin B and its trans-activation by an endogenous asparaginyl endopeptidase. Mol Biochem Parasitol. 2003; 131(1):65–75. PMID: 12967713

35. Srp J, Nussbaumerova M, Horn M, Mares M. Digestive proteolysis in the Colorado potato beetle, Leptinotarsa decemlineata: Activity-based profiling and imaging of a multipeptidase network. Insect Biochem Mol Biol. 2016; 78:1–11. https://doi.org/10.1016/j.ibmb.2016.08.004 PMID: 27539253.

36. Horn M, Nussbaumerova M, Sanda M, Kovarova Z, Srba J, Franta Z, et al. Hemoglobin Digestion in Blood-Feeding Ticks: Mapping a Multipeptidase Pathway by Functional Proteomics. Chemistry & Biology. 2009; 16(10):1053–63. https://doi.org/10.1016/j.chembiol.2009.09.009 WOS:000271894000008. PMID: 19875079

37. Fajtova P, Stefanic S, Hradilek M, Dvorak J, Vondrasek J, Jilkova A, et al. Prolyl Oligopeptidase from the Blood Fluke Schistosoma mansoni: From Functional Analysis to Anti-schistosomal Inhibitors. Plos Neglected Tropical Diseases. 2015; 9(6). https://doi.org/10.1371/journal.pntd.0003827 WOS:000357398100031. PMID: 26039195

38. Doleckova-Maresova L, Pavlik M, Horn M, Mares M. De novo design of alpha-amylase inhibitor: A small linear mimetic of macromolecular proteinaceous ligands. Chemistry & Biology. 2005; 12(12):1349–57. https://doi.org/10.1016/j.chembiol.2005.10.005 WOS:000234358400013. PMID: 16356852

39. Choe Y, Leonetti F, Greenbaum DC, Lecaille F, Bogyo M, Bromme D, et al. Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. J Biol Chem. 2006; 281(18):12824–32. https://doi.org/10.1074/jbc.M513331200 PMID: 16520377

40. Colaert N, Helsens K, Martens L, Vandekerckhove J, Gevaert K. Improved visualization of protein consensus sequences by iceLogo. Nat Methods. 2009; 6(11):786–7. https://doi.org/10.1038/nmeth1109-786 PMID: 19876014.

41. Adams JC, Tucker RP. The thrombospondin type 1 repeat (TSR) superfamily: diverse proteins with related roles in neuronal development. Dev Dyn. 2000; 218(2):280–99. Epub 2000/06/08. https://doi.org/10.1002/(SICI)1097-0177(200006)218:2<280::AID-DVDY4>3.0.CO;2-0 PMID: 10842357.

42. Chen H, Herndon ME, Lawler J. The cell biology of thrombospondin-1. Matrix Biol. 2000; 19:597–614. Epub 2000/12/05. PMID: 11102749.

43. Tan K, Duquette M, Liu JH, Dong Y, Zhang R, Joachimiak A, et al. Crystal structure of the TSP-1 type 1 repeats: A novel layered fold and its biological implication. Journal of Cell Biology. 2002; 159:373–82. https://doi.org/10.1083/jcb.200206062 PMID: 12391027.

44. Tan K, Duquette M, Liu JH, Dong Y, Zhang R, Joachimiak A, et al. Crystal structure of the TSP-1 type 1 repeats: a novel layered fold and its biological implication. J Cell Biol. 2002; 159(2):373–82. https://doi.org/10.1083/jcb.200206062 PMID: 12391027; PubMed Central PMCID: PMCPMC2173040.

45. Huber R, Bode W. Structural Basis of the Activation, Action and Inhibition of Trypsin. H-S Z Physiol Chem. 1979; 360(4):489–. WOS:A1979GS98300001.

46. Rawlings ND, Waller M, Barrett AJ, Bateman A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 2014; 42(Database issue):D503–D9. gkt953 [pii]; https://doi.org/10.1093/nar/gkt953 PMID: 24157837

47. Young ND, Hall RS, Jex AR, Cantacessi C, Gasser RB. Elucidating the transcriptome of Fasciola hepatica—a key to fundamental and biotechnological discoveries for a neglected parasite. Biotechnol Adv. 2010; 28(2):222–31. https://doi.org/10.1016/j.biotechadv.2009.12.003 PMID: 20006979.

48. Perona JJ, Craik CS. Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold. J Biol Chem. 1997; 272(48):29987–90. PMID: 9374470.

49. Dobo J, Harmat V, Beinrohr L, Sebestyen E, Zavodszky P, Gal P. MASP-1, a promiscuous complement protease: structure of its catalytic region reveals the basis of its broad specificity. J Immunol. 2009; 183(2):1207–14. https://doi.org/10.4049/jimmunol.0901141 PMID: 19564340.

50. Harris JL, Backes BJ, Leonetti F, Mahrus S, Ellman JA, Craik CS. Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. Proc Natl Acad Sci U S A. 2000; 97(14):7754–9. https://doi.org/10.1073/pnas.140132697 [doi];140132697 [pii]. PMID: 10869434

51. O'Donoghue AJ, Eroy-Reveles AA, Knudsen GM, Ingram J, Zhou M, Statnekov JB, et al. Global identification of peptidase specificity by multiplex substrate profiling. Nat Methods. 2012; 9(11):1095–100. nmeth.2182 [pii]; https://doi.org/10.1038/nmeth.2182 PMID: 23023596

52. Yang Y, Wen Y, Cai YN, Vallee I, Boireau P, Liu MY, et al. Serine proteases of parasitic helminths. Korean J Parasitol. 2015; 53(1):1–11. https://doi.org/10.3347/kjp.2015.53.1.1 PMID: 25748703; PubMed Central PMCID: PMCPMC4384789.

53. Dvorak J, Mashiyama ST, Braschi S, Sajid M, Knudsen GM, Hansell E, et al. Differential use of protease families for invasion by schistosome cercariae. Biochimie. 2008; 90(2):345–58. https://doi.org/10.1016/j.biochi.2007.08.013 PMID: 17936488

54. Walsh PN, Ahmad SS. Proteases in blood clotting. Essays Biochem. 2002; 38:95–111. PMID: 12463164.

55. Harder A, Andrews P, Thomas H. Praziquantel: mode of action. Biochem Soc Trans. 1987; 15(1):68–70. PMID: 3493930.

56. Lorenzo C, Salinas G, Brugnini A, Wernstedt C, Hellman U, Gonzalez-Sapienza G. Echinococcus granulosus antigen 5 is closely related to proteases of the trypsin family. Biochem J. 2003; 369(Pt 1):191–8. https://doi.org/10.1042/BJ20021402 PMID: 12358601; PubMed Central PMCID: PMCPMC1223071.

57. Rueda A, Sifuentes C, Gilman RH, Gutierrez AH, Pina R, Chile N, et al. TsAg5, a Taenia solium cysticercus protein with a marginal trypsin-like activity in the diagnosis of human neurocysticercosis. Mol Biochem Parasit. 2011; 180(2):115–9. https://doi.org/10.1016/j.molbiopara.2011.08.003 WOS:000297235900007. PMID: 21893105

58. Li Y, Xu H, Chen J, Gan W, Wu W, Wu W, et al. Gene cloning, expression, and localization of antigen 5 in the life cycle of Echinococcus granulosus. Parasitol Res. 2012; 110(6):2315–23. https://doi.org/10.1007/s00436-011-2766-9 PMID: 22200957.

59. Harnett W. The anthelmintic action of praziquantel. Parasitol Today. 1988; 4(5):144–6. PMID: 15463071.

60. Becker B, Mehlhorn H, Andrews P, Thomas H, Eckert J. Light and electron microscopic studies on the effect of praziquantel on Schistosoma mansoni, Dicrocoelium dendriticum, and Fasciola hepatica (Trematoda) in vitro. Z Parasitenkd. 1980; 63(2):113–28. PMID: 7456640.

61. Lightowlers MW, Liu DY, Haralambous A, Rickard MD. Subunit composition and specificity of the major cyst fluid antigens of Echinococcus granulosus. Mol Biochem Parasitol. 1989; 37(2):171–82. PMID: 2481826.

62. Sotillo J, Pearson M, Potriquet J, Becker L, Pickering D, Mulvenna J, et al. Extracellular vesicles secreted by Schistosoma mansoni contain protein vaccine candidates. Int J Parasitol. 2016; 46(1):1–5. Epub 2015/10/16. https://doi.org/10.1016/j.ijpara.2015.09.002 PMID: 26460238.

63. Zhu L, Liu J, Dao J, Lu K, Li H, Gu H, et al. Molecular characterization of S. japonicum exosome-like vesicles reveals their regulatory roles in parasite-host interactions. Sci Rep. 2016; 6:25885. Epub 2016/05/14. https://doi.org/10.1038/srep25885 PMID: 27172881; PubMed Central PMCID: PMCPMC4865838.

64. Samoil V, Dagenais M, Ganapathy V, Aldridge J, Glebov A, Jardim A, et al. Vesicle-based secretion in schistosomes: Analysis of protein and microRNA (miRNA) content of exosome-like vesicles derived from Schistosoma mansoni. Sci Rep. 2018; 8(1):3286. Epub 2018/02/21. https://doi.org/10.1038/s41598-018-21587-4 PMID: 29459722; PubMed Central PMCID: PMCPMC5818524.

65. Baird TT, Craik CS. Trypsin. Handbook of Proteolytic Enzymes, Vols 1 and 2, 3rd Edition. 2013:2594–600. https://doi.org/10.1016/b978-0-12-382219-2.00575–5 WOS:000328545104016.

66. Basch PF. Schistosomes: development, reproduction, and host relations. New York: Oxford University Press; 1991. vii, 248 p. p.

67. Mebius MM, van Genderen PJ, Urbanus RT, Tielens AG, de Groot PG, van Hellemond JJ. Interference with the host haemostatic system by schistosomes. PLoS Pathog. 2013; 9(12):e1003781. https://doi.org/10.1371/journal.ppat.1003781 PMID: 24385897; PubMed Central PMCID: PMCPMC3873443.

68. Campbell DJ. The renin-angiotensin and the kallikrein-kinin systems. Int J Biochem Cell Biol. 2003; 35(6):784–91. S1357272502002625 [pii]. PMID: 12676165

69. Maurer M, Bader M, Bas M, Bossi F, Cicardi M, Cugno M, et al. New topics in bradykinin research. Allergy. 2011; 66(11):1397–406. https://doi.org/10.1111/j.1398-9995.2011.02686.x PMID: 21859431.

70. Da'dara AA, Skelly PJ. Schistosomes versus platelets. Thromb Res. 2014; 134(6):1176–81. https://doi.org/10.1016/j.thromres.2014.09.032 PMID: 25294585.

71. Figueiredo BC, Da'dara AA, Oliveira SC, Skelly PJ. Schistosomes Enhance Plasminogen Activation: The Role of Tegumental Enolase. PLoS Pathog. 2015; 11(12):e1005335. https://doi.org/10.1371/journal.ppat.1005335 PMID: 26658895; PubMed Central PMCID: PMCPMC4676649.

72. Boose JA, Kuismanen E, Gerard R, Sambrook J, Gething MJ. The single-chain form of tissue-type plasminogen activator has catalytic activity: studies with a mutant enzyme that lacks the cleavage site. Biochemistry. 1989; 28(2):635–43. PMID: 2496749.

73. Brown NJ, Gainer JV, Stein CM, Vaughan DE. Bradykinin stimulates tissue plasminogen activator release in human vasculature. Hypertension. 1999; 33(6):1431–5. PMID: 10373228.

74. Wang Q, Da'dara AA, Skelly PJ. The human blood parasite Schistosoma mansoni expresses extracellular tegumental calpains that cleave the blood clotting protein fibronectin. Sci Rep. 2017; 7(1):12912. Epub 2017/10/12. https://doi.org/10.1038/s41598-017-13141-5 PMID: 29018227; PubMed Central PMCID: PMCPMC5635006.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Prolyl Oligopeptidase from the Blood Fluke *Schistosoma mansoni*: From Functional Analysis to Anti-schistosomal Inhibitors

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Abstract

Background

Blood flukes of the genus *Schistosoma* cause schistosomiasis, a parasitic disease that infects over 240 million people worldwide, and for which there is a need to identify new targets for chemotherapeutic interventions. Our research is focused on *Schistosoma mansoni* prolyl oligopeptidase (SmPOP) from the serine peptidase family S9, which has not been investigated in detail in trematodes.

Methodology/Principal Findings

We demonstrate that SmPOP is expressed in adult worms and schistosomula in an enzymatically active form. By immunofluorescence microscopy, SmPOP is localized in the tegument and parenchyma of both developmental stages. Recombinant SmPOP was produced in *Escherichia coli* and its active site specificity investigated using synthetic substrate and inhibitor libraries, and by homology modeling. SmPOP is a true oligopeptidase that hydrolyzes peptide (but not protein) substrates with a strict specificity for Pro at P1. The inhibition profile is analogous to those for mammalian POPs. Both the recombinant enzyme and live worms cleave host vasoregulatory, proline-containing hormones such as angiotensin I and bradykinin. Finally, we designed nanomolar inhibitors of SmPOP that induce deleterious phenotypes in cultured schistosomes.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions/Significance

We provide the first localization and functional analysis of SmPOP together with chemical tools for measuring its activity. We briefly discuss the notion that SmPOP, operating at the host-parasite interface to cleave host bioactive peptides, may contribute to the survival of the parasite. If substantiated, SmPOP could be a new target for the development of anti-schistosomal drugs.

Author Summary

Schistosomiasis (bilharzia) is a major global health problem caused by the schistosome flatworm which lives in the bloodstream. Treatment and control of the disease relies on a single drug, and should resistance emerge, there would be increased pressure to discover new drug targets. Proteolytic enzymes are fundamental to the survival of parasites, and, hence, are attractive targets for drug intervention. Oligopeptidases from the S9 family are known virulence factors for protozoan trypanosomatids but have yet to be studied in parasitic flukes. We, therefore, investigated prolyl oligopeptidase in *Schistosoma mansoni* (SmPOP) and found that it is expressed in those developmental stages that infect humans. We provide a comprehensive analysis of the peptidase's expression, localization and functional biochemical properties. Interestingly, SmPOP, which is found in the tegument and parenchyma of the parasite, can cleave blood peptides involved in vasoregulation and we discuss how this ability may aid the parasite's survival. Finally, we designed potent inhibitors of SmPOP that cause deleterious changes in cultured parasites. We conclude that SmPOP is important for parasite survival and may be a potential target for the development of anti-schistosomal drugs.

Introduction

Schistosomiasis, also known as bilharzia, is caused by blood flukes of the genus *Schistosoma* with approximately 240 million people infected [1]. Three species of schistosome principally infect humans: *Schistosoma haematobium*, which causes urinary schistosomiasis, and *S. japonicum* and *S. mansoni*, which cause intestinal schistosomiasis [2]. Adult schistosomes can reside for decades as pairs in the veins surrounding the bladder or in mesenteric and the portal veins, and produce hundreds of eggs per day [3]. Morbidity arises from immuno-pathological reactions to and entrapment of schistosome eggs in various tissues [4]. Disease symptoms include spleno- and hepatomegaly, periportal fibrosis and hypertension, and urinary obstruction. Bladder carcinoma, sterility, malnutrition, and developmental retardation are common [3]. Infections can last a lifetime [5].

In the absence of a vaccine [6], control and treatment of schistosomiasis rely on a single drug, praziquantel, and the possibility of emergent drug resistance is a constant concern [7,8]. Accordingly, there is a continued impetus to identify new schistosome drug targets and chemotherapeutically active anti-schistosomals [8,9].

Proteolytic enzymes (peptidases) of schistosomes are attractive drug targets as they operate at the host–parasite interface, where they may facilitate parasite invasion, migration, nutrition and immune evasion [10–12]. Most studies concerning schistosome peptidases have focused on either the serine peptidase called cercarial elastase that facilitates penetration of the human

host by some schistosome species [13] or on those cysteine and aspartic peptidases that contribute to the digestion of the blood meal [14,15]. Among the latter, the digestive cathepsin B of *S. mansoni*, known as SmCB1, has been validated in a murine model of *S. mansoni* infection as a molecular target for therapy [9,16] and small molecule inhibitors of SmCB1 are under consideration as potential drug leads [16–19]. Other peptidase groups of schistosomes are less studied [12], including post-proline cleaving peptidases. This work focused on a *S. mansoni* prolyl oligopeptidase.

Prolyl oligopeptidases (POPs, also called prolyl endopeptidases) are approximately 70–80 kDa and belong to the S9 family of serine peptidases [20]. POPs cleave internal peptide bonds on the C-terminal side of proline residues and are found in plants, bacteria, fungi, protozoa, invertebrates and vertebrates [21]. For parasites, the best characterized POP is Tc80 in the infective trypomastigote stage of *Trypanosoma cruzi*, the causative agent of Chagas disease [22]. Tc80 seems to be involved in the parasite invasion as inhibition of Tc80 prevents parasite entry into host cells [23]. Accordingly, Tc80 is under investigation as a potential drug target [23,24].

In this report, we identified and functionally characterized the prolyl oligopeptidase from *S. mansoni* (SmPOP). We demonstrate that enzymatically active SmPOP is produced in several developmental stages and localized to the tegument and parenchyma of the parasite. We characterized in detail the biochemical activity of recombinant and native SmPOP, and designed nanomolar inhibitors of SmPOP that derange schistosomes maintained in culture. The data suggest that SmPOP is important to parasite survival and is, thus, a potential target for the development of anti-schistosomal therapeutics.

Materials and Methods

Ethics statement

All animal procedures were performed at the UCSF, USA, in accordance with protocol (AN107779–01) approved by the UCSF Institutional Animal Care and Use Committee (IACUC) as required by the Federal Animal Welfare Act and the National Institutes of Health Public Health Service Policy on Humane Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/references/phspol.htm).

Schistosome material

S. mansoni (a Puerto Rican isolate) was kept in the University of California San Francisco (UCSF) laboratory by cycling between the intermediate snail host, *Biomphalaria glabrata*, and female golden Syrian hamsters (infected at 3–5 weeks old), as the definitive host. Hamsters are infected by subcutaneous injection of 800 cercariae and sacrificed 6–7 weeks post-infection by intra-peritoneal injection of pentobarbital (50 mg/kg). Adults, eggs and miracidia were then isolated as described previously [25,26]. Free-swimming cercariae were obtained from water containing infection-patent *Biomphalaria* to 'shed' under a bright light. Cercariae were chilled over ice. Newly transformed schistosomula (NTS) were prepared by mechanically transforming cercariae [26,27] and cultivated in a Basch Medium 169 [28] containing 5% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin for 5 days at 37°C under a 5% CO_2 atmosphere. Daughter sporocysts were isolated by excision of hepato-pancreases from infected *B. glabrata* snails.

Isolation of mRNA, cDNA synthesis and qRT-PCR

Adult worms, eggs, miracidia, daughter sporocysts, cercariae and NTS were collected, washed three times in 1.5 mL PBS, re-suspended in 500 μL Trizol reagent (Invitrogen) and processed

as described previously [26]. Single-stranded cDNA was synthesized from total RNA by SuperScript III reverse transcriptase (Life Technologies) and an oligo dT_{18} primer. The final cDNA product was purified and stored at -20°C.

The gene expression profile of the SmPOP was assessed using reverse transcription- quantitative PCR (RT-qPCR). The following primers were used: forward 5'-CATTCGTGGTGGAGGAGAAT-3' and reverse 5'- CGCATACTGGAACTTGAGCA -3'. The primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/) and their efficiency was evaluated as described previously [25,26]. The reactions, containing SYBR Green I Mastermix (Eurogentech), were prepared in a final volume of 25 μL in 96-well plates (Roche). The amplification profile consisted of an initial hot start (95°C for 10 min) followed by 40 cycles comprising 95°C for 30 s, 55°C for 60 s and 72°C for 60 s, and ending with a single cycle of 95°C for 60 s, 55°C for 30 s and 95°C for 30 s. The PCR reactions were performed in duplicate for each cDNA sample. At least one biological replicate, *i.e.*, samples from a different RNA isolation, was performed. The analysis of the cycle threshold for each target was carried out as described [25,26] employing *S. mansoni* cytochrome c oxidase I (SmCOX I, GenBank AF216698) [29] as the sample-normalizing gene transcript. Transcript levels were expressed as log functions and as a percentage relative to that of SmCOX I in order to compare expression patterns.

Expression and purification of recombinant SmPOP

The single gene encoding SmPOP (SchistoDB code: Smp_213240) was identified in the *S. mansoni* genome database [12] (*S. mansoni* GeneDB available at http://www.genedb.org/Homepage/Smansoni) via a protein BLAST search with the amino acid sequences of human and porcine prolyl oligopeptidases (GenBank accession numbers P48147 and P23687, respectively) as queries. The same search in the *S. japonicum* and *S. haematobium* genome databases [30,31] identified SmPOP orthologs with 88% and 95% identity, respectively (*S. japonicum*: GeneDB Sjp_0080730.1, GenBank AAX26405; *S. haematobium*: HelmDB Shae8836338, GenBank KGB33720).

The Champion pET directional expression kit (Life Technologies) was selected for expression of the SmPOP gene. The 2139 bp ORF was amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs) from adult schistosome cDNA using specific forward (5′-caccAT GGAGCATACCAGTATCAACTATCC-3′) and reverse (5′-TTCTTTCCATGTGAGT GACATT-3′) primers. The PCR product was cloned into the expression vector pET101/D-T OPO (Invitrogen) and verified by DNA sequencing. Recombinant SmPOP (rSmPOP) with a C-terminal His_6-tag was produced in *E. coli* BL21(DE3) by induction in LB broth medium containing 0.5 mM IPTG for 16 h at 16°C. Soluble rSmPOP was purified from the bacterial lysate using Ni^{2+} chelating chromatography (Hi-Trap IMAC FF column, GE Healthcare Life Sciences) under native conditions. The bound rSmPOP was eluted using a linear gradient of 0.01–0.5 M imidazole. The preparation was buffer-exchanged into 20 mM Tris-HCl, pH 8.0, using an Amicon Ultracel-30k ultrafiltration device (Millipore). rSmPOP was subsequently purified by FPLC over a Mono Q HR 5/5 column (GE Healthcare Life Sciences) equilibrated in 20 mM Tris-HCl, pH 8.0, and eluted using a linear gradient of 0–1 M NaCl in the same buffer. The purification process was monitored by a kinetic assay incorporating the peptidyl fluorogenic substrate, benzyloxycarbonyl (Z)-Gly-Pro-7-amino-4-methylcoumarin (AMC), and by SDS-PAGE. The preparation was concentrated and desalted into 20 mM Tris-HCl, pH 8.0, using an Amicon Ultracel-30k. The typical yield was approximately 3 mg of rSmPOP from 1 L of culture medium.

Preparation of schistosome extracts

Soluble protein extracts (0.2–3 mg protein/mL) from *S. mansoni* adults, miracidia, cercariae, eggs and NTS were prepared by homogenization in 50 mM Tris-HCl, pH 8.0, containing 1%

CHAPS, 1 mM EDTA, 1 μM pepstatin and 10 μM E-64 in an ice bath. The extracts were cleared by centrifugation (16000 *g* at 4°C for 10 min,), ultra-filtered using a 0.22 μm Ultrafree-MC device (Millipore) and stored at -80°C.

Preparation of antibodies and immunoblotting

Specific polyclonal antibodies (Moravian Biotechnology) were generated in rabbits against the purified rSmPOP antigen using 50 μg of rSmPOP in Freund's incomplete adjuvant and applied three times three weeks apart. IgG was isolated from the serum by affinity chromatography with a HiTrap Protein A column (GE Healthcare Life Sciences) according to the manufacturer's protocol.

For immunoblotting, adult schistosome homogenate (30 μg protein) and rSmPOP (1 μg) were resolved by SDS-PAGE (15% polyacrylamide gel) under reducing conditions and transferred onto a PVDF membrane. The membrane was blocked 16 h in 10% non-fat milk in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.1% Tween (TTBS). The membrane was then washed three times in TTBS and incubated for 1 h with anti-SmPOP polyclonal IgG diluted 1:1000 in TTBS. After washing in TTBS, the membrane was incubated for 1 h with goat horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich, catalog number A6154) at a dilution of 1:20000. After washing in TTBS, the membrane was developed with SuperSignal West Femto Chemiluminescent Substrate (Pierce) and imaged using an ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences).

Immunofluorescence microscopy

For sample preparation, adult *S. mansoni* worms were washed three times in PBS and fixed either in acetone (75% acetone in ethanol) at -20°C for 10 min or 4% formaldehyde in PBS at 25°C for 45 min. The samples were then rinsed with PBS and incubated in a 30% sucrose solution at 4°C for 16 h. The worms were placed in cryofixation molds and the sucrose solution was replaced with Optimal Cutting Temperature (OCT) medium (CellPath Ltd). The molds were placed over dry ice to freeze and the frozen blocks then stored at -80°C. The OCT-embedded worm samples were sectioned with a cryotome (Cryostat 2800 Frigocout, Cambridge Instruments). Sections of ~7 μm were air-dried and further processed for immunostaining.

Sections were rehydrated in PBS and fixed again either with formaldehyde or cold acetone as described above. The formaldehyde-fixed samples were further blocked in 100 mM glycine at 22°C for 20 min, followed by 2% BSA in PBS at 4°C for 16 h. Working solutions of primary and secondary antibodies were prepared in PBS containing 2% BSA; rabbit polyclonal anti-SmPOP IgG was diluted 1:900 and anti-rabbit IgG Alexa 594-conjugated secondary antibody (Molecular Probes) was diluted 1:200. The antibodies were incubated at 25°C on the sections for 45 min with three washes between the primary and secondary antibody incubations, and four washes after the secondary-antibody incubation (the fourth wash contained DAPI at 1 μg/mL for nuclear staining).

NTS samples were fixed in 4% formaldehyde in PBS at 4°C for 16 h. After fixation, they were washed 3 times in PBS at 25°C for 10 min and subsequently blocked with 100 mM glycine at 25°C for 20 min. Samples were permeabilized with 0.2% Triton X-100 in PBS for 40 min at 25°C and blocked with 2% BSA in PBS for 16 h at 4°C. The antibody diluent contained 0.1% Triton X-100, 0.1% BSA and 0.2% NaN_3. Primary and secondary antibody solutions were incubated for 24 h with four washes of diluent over a 10 h period (the fourth wash contained DAPI at 1 μg/mL for nuclear staining).

Sections of adults and whole-worm preparations of NTS were embedded in Mowiol (Sigma-Aldrich) and visualized using a Leica SP2 AOBS confocal laser scanning microscope (Leica

Microsystems) and a 20x oil immersion objective. Appropriate lighting settings were determined using control slides probed with preimmune serum to define the background signal threshold. Image stacks of optical sections were further processed using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging).

Preparation of substrates and inhibitors

Fluorescence resonance energy transfer (FRET) substrates containing o-aminobenzoic acid (Abz) as the fluorescent group and p-nitro-phenylalanine (NPh) as the quencher acceptor were synthesized as peptidyl amides by Fmoc solid phase chemistry in an ABI 433A peptide synthesizer (Applied Biosystems) as described previously[16,32].

Substrates containing the fluorogenic group, 7-amino-4-carbamoylmethylcoumarin (ACC), were synthesized in the format Z-Xaa-Pro-ACC, with proteinogenic amino acids (except for cysteine) at the Xaa position, as described previously [33].

The inhibitors Z-Ala-Pro-chloromethyl ketone (CMK) and Z-Arg-Pro-CMK were prepared from the peptides Z-Ala-Pro-OH and Z-Arg(Pbf)-Pro-OH, respectively, according to the described procedure [34]. Z-Ala-Pro-OH and Z-Arg(Pbf)-Pro-OH were synthesized on solid phase using 2-chlorotritylchloride resin (Iris Biotech). Z-Xaa-Pro-CHO (CHO, aldehyde) inhibitors, where Xaa is Gly, Ala, Tyr, Arg or Lys, were synthesized on solid phase using H-Thr-Gly-NovaSyn TG resin (Merck) as described [35]. All of the substrates and inhibitors were purified by reverse-phase (RP)-HPLC over a C18 column using a TFA/acetonitrile system and characterized by electrospray ionization mass spectrometry on an LCQ Classic Finnigan Mat device (Thermo Finnigan).

The substrates Z-Gly-Pro-AMC, Succinyl (Suc)-Gly-Pro-Leu-Gly-Pro-AMC, Lys-Pro-AMC, Gly-Pro-AMC and Pro-AMC were purchased from Bachem. The POP inhibitors Y-29794 oxalate and Z-Pro-Pro-CHO were purchased from Santa Cruz Biotechnology, and SUAM 14746 from PeptaNova.

Kinetic POP activity and inhibition assays

Assays were performed in triplicate in black, flat-bottomed, 96-well microplates (Nunc) in a total volume of 100 μL at 37°C. Z-Gly-Pro-AMC was used as substrate at a 50 μM final concentration. rSmPOP (50–100 ng), human POP (25–50 ng; Sigma-Aldrich, catalog number O9515) or schistosome homogenates (1–5 μg of protein) were pre-incubated for 10 min at 37°C in 80 μL of 0.1 M sodium phosphate, pH 8.0, containing 0.1% PEG 6000. Substrate (20 μL in the same buffer) was added to a final concentration of 50 μM. Hydrolytic activity was measured continuously in an Infinite M1000 microplate reader (Tecan) at the excitation and emission wavelengths of 360 and 465 nm, respectively. The pH profile of the activity was determined in 100 mM citrate phosphate (pH range 5.5–8.0), 100 mM Tris-HCl (pH range 8.0–9.0) and 100 mM sodium borate (pH range 9.0–10.0). For inhibition measurements, inhibitors were added to the 80 μL pre-incubation solution at a final concentrations of 0 to125 μM for 10 min and the reaction was initiated by the addition of the substrate. IC_{50} values were determined by nonlinear regression using the GraFit software (Erithacus Software). SmPOP activity in homogenates was measured in the presence of 10 μM E-64 to prevent undesired proteolysis by cysteine peptidases that contribute significant proteolytic activity in worm extracts [36]. POP activity was also measured with ACC and FRET substrates at excitation/emission wavelengths of 380/460 nm and 320/420 nm, respectively. Stock solutions of substrates and inhibitors (10 mM) were prepared in DMSO and the final assays concentration of DMSO was 1.5%.

Interaction of rSmPOP with protein substrates

rSmPOP (0.7 μg) was incubated at 37°C for 16 h with 100 μg of human hemoglobin, human serum albumin, human collagens type I and IV (Sigma-Aldrich, catalog numbers H7379, A3782, C7774 and C7521 respectively) in 100 mM Tris-HCl, pH 8.0, in a final volume of 50 μL. After incubation, a 10 μL sample was resolved by 15% SDS-PAGE or Tricine-SDS-PAGE and stained with Coomassie Brilliant Blue G250.

Hydrolysis of peptide hormones and neuropeptides by rSmPOP

The following synthetic analogues of human bioactive peptides were analyzed: angiotensin II (Sigma, catalog number A9525), angiotensin I, bradykinin, luteinizing-hormone-releasing hormone (LHRH), α-melanocyte-stimulating hormone (α-MSH), neurotensin, oxytocin, substance P, and vassopresin (all Bachem, catalog numbers H-1680, H-1970, H-6728, H-1075, H-4435, H-2510, H-1890 and H-1780, respectively). Stock solutions of peptides (10 mM) were prepared in water. rSmPOP (0.7 μg) was incubated at 37°C for 16 h with 25 nmol of peptide in 0.1 M Tris-HCl, pH 8.0, in a total volume of 50 μL. The reaction was stopped by adding TFA to a final concentration of 1%. The resulting fragments were purified by RP-HPLC over a C18 column (Vydac, 25 x 0.46 cm) using a TFA/acetonitrile system and characterized by electrospray ionization mass spectrometry on an LCQ Classic Finnigan Mat device (Thermo Finnigan).

Hydrolysis of peptide hormones and neuropeptides by cultured schistosomes

Five adult schistosome pairs were placed into clear, 24-well, flat-bottom plates (Costar) containing 500 μL Basch Medium 199 [28], supplemented with 2.5% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Human angiotensin I or bradykinin in 5 μL water was added to a final concentration of 100 μM and the incubation continued for 16 h at 37°C under a 5% CO_2 atmosphere. In control experiments, the peptides were cultivated in the same system in the absence of schistosomes. After incubation, the samples were ZipTiped and the resulting fragments were analyzed using MALDI-TOF performed on an UltrafleXtreme (Bruker Daltonik) operated in reflectron mode with an acceleration voltage of 25 kV and a pulsed ion extraction of 120 ns. Desorption and ionization were achieved using a Smartbeam II laser. α-Cyano-4-hydroxycinnamic acid was used as a matrix. The data were acquired from m/z 220 to 3700 and analyzed with the FlexAnalysis 3.3 software (Bruker Daltonik). The mass spectra were externally calibrated using a Peptide Calibration Standard I (Bruker Daltonik) and averaged from 3000 laser shots.

Fluorescence SmPOP activity assay with cultured schistosomes

Adult worms (3 pairs) or approximately 150 NTS were incubated at 37°C and 5% CO_2 for 2 days in 200 μL of Basch Medium 169 containing 5% FBS, 100 units/mL of penicillin and 100 μg/mL using black clear bottomed 96-well microplates (Costar). After incubation, half of the medium (100 μl) was transferred to an empty well leaving the parasites in the remaining half. Then SmPOP activity was measured in both wells upon the addition of 20 μL of Z-Gly-Pro-AMC (prepared as a 250 μM stock in Basch Medium 169) and in the presence or absence of 1 μM of the POP inhibitor, Z-Ala-Pro-CMK. Controls contained medium alone.

Molecular modeling of SmPOP

A spatial model of SmPOP was constructed by homology modeling as described previously [17]. Briefly, the X-ray structure of porcine POP in complex with the inhibitor Z-Pro-Pro-CHO (PDB

entry: 1QFS) was used as a template. The homology module of the MOE program (Chemical Computing Group) was used for the modeling of the SmPOP structure. The inhibitor conformation was refined by applying the LigX module of the MOE for the optimization procedure and its final binding mode was selected by the best-fit model based on the London dG scoring function and the generalized Born method [37]. Molecular images were generated with UCSF Chimera (http://www.cgl.ucsf.edu/chimera/).

Parasite assay and phenotype scoring

NTS (200–300 parasites) were incubated in 200 μL of Basch Medium 169 and supplements, as described above. Inhibitors were added at final concentrations of 1 or 10 μM (0.5% DMSO final) and the incubations continued for 4 days. Grading of phenotypic responses arising as a function of time and concentration was modified after Jilkova *et al*. [16]: Grade I, dead NTS by 2 days of culture at 10 μM and dying/dead NTS by 3 days at 1 μM. Grade II, dead NTS by 3 days at 10 μM and round/dark/dying by 3 days at 1 μM; Grade III, round/dark by 3 days at 1 and 10 μM concentrations (S1 Fig). 'Dead' was adjudicated as the loss of normal shape and the lack of movement often accompanied by obvious internal disruptions. 'Dying' was similar to death except that movement was detectable. Otherwise, the terms 'round/dark' were used to indicate less severe but obvious changes in the parasites relative to DMSO controls.

Results

SmPOP is homologous to prolyl oligopeptidases from various parasites

A protein BLAST analysis of the *S. mansoni* genome database [12,38] using mammalian prolyl oligopeptidases as queries identified a gene ortholog (SmPOP), Smp_213240, located on the sex-determining Z/W chromosomal pair. SmPOP cDNA was cloned, sequenced, and the sequence was deposited into the GenBank as KF956809. The blast analysis did not identify other gene isoforms. The SmPOP open reading frame consists of 2,139 bp that encodes a protein of 712 amino acid residues with a calculated molecular mass of 82 kDa. No signal/leader peptide was predicted for the sequence. SmPOP has about 50% identity with human and porcine POPs (S1 Table) and belongs to the S9 family of serine peptidases (S2 Fig). SmPOP has the characteristic domain composition of mammalian POPs, consisting of N-terminal, β-propeller and peptidase domains. The peptidase domain of SmPOP has a catalytic triad in the order of Ser556, Asp643 and His682, which is typical of POPs and other S9 family peptidases [39]. In addition, the regions surrounding the catalytic-triad residues have the most notable sequence identity. A phylogenetic tree constructed for prolyl oligopeptidases of animal, plant, protozoan and bacterial origin (S3 Fig) demonstrates that SmPOP clusters with other trematode and nematode POPs. This monophyletic group is well separated from other clades.

S. mansoni developmental stages express active POP

Messenger RNA transcript levels for SmPOP were evaluated in eggs, miracidia, daughter sporocysts, cercariae, NTS and adults using qRT-PCR (Fig 1A). The expression of SmPOP was recorded in eggs, daughter sporocysts, NTS and adult schistosomes (in the range of 4–12% of the expression of the validated reference gene, SmCOX I [26]). In miracidia and cercariae, expression was below 1% of the SmCOX I level (Fig 1A).

At the protein level, SmPOP enzymatic activity in soluble extracts of various developmental stages was determined in a kinetic assay using the fluorogenic substrate, Z-Gly-Pro-AMC, which is specific for prolyl oligopeptidases. The measured activities were further authenticated as being due to a prolyl oligopeptidase by their sensitivity to Z-Pro-Pro-CHO, a selective

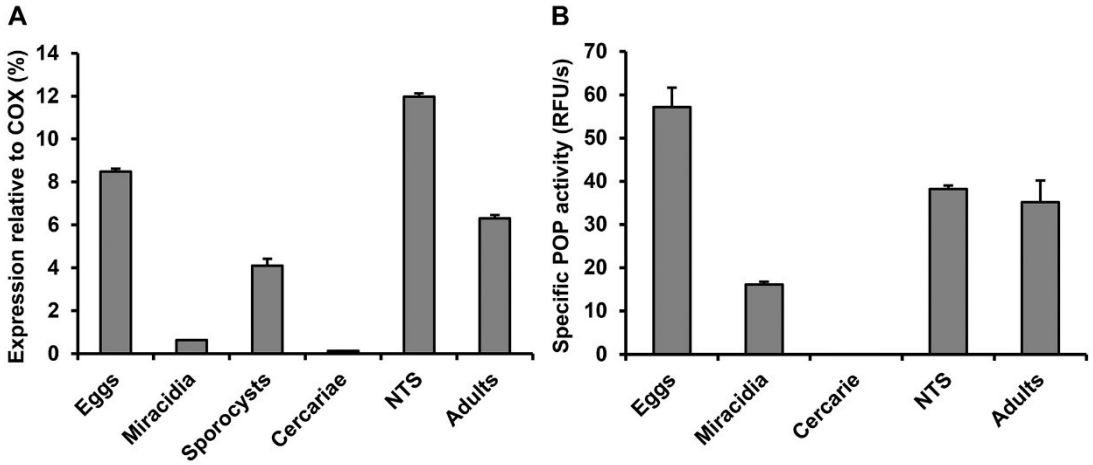

Fig 1. Activity and transcriptional profiling of SmPOP in the developmental stages of *S. mansoni*. (A) The expression of SmPOP was evaluated by quantitative RT-PCR. mRNA transcriptional levels are presented as the percentage of expression relative to the constitutively expressed *S. mansoni* cytochrome oxidase I (SmCOX I). The mean values ± S.D. of three replicates are given. (B) SmPOP activities were measured in protein extracts of the developmental stages (except sporocysts not available in sufficient amount and purity) using a kinetic assay with the fluorogenic substrate Z-Gly-Pro-AMC at pH 8.0. POP activities (sensitive to inhibition by the specific POP inhibitor Z-Pro-Pro-CHO) are expressed in relative fluorescence units (RFU/s) and normalized to protein content.

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small-molecule inhibitor of prolyl oligopeptidases [40]. Prominent SmPOP activity was measured in the homogenates of eggs, NTS and adults, whereas weak activity was measured in miracidial homogenates; no activity was detected in cercariae (Fig 1B).

Overall, active SmPOP is expressed in the *S. mansoni* developmental stages that live in the human host and the activity profile is consistent with that for mRNA expression. In addition, the presence of SmPOP was confirmed in the protein homogenate of adult *S. mansoni* by mass spectrometry proteomics (S2 Table).

SmPOP cleaves proline-containing neuropeptides and oligopeptide hormones of the host

Recombinant SmPOP (rSmPOP) was expressed in *E. coli* as a soluble and catalytically active enzyme. rSmPOP was purified to homogeneity by a combination of metal-affinity chromatography and ion-exchange chromatography, and subsequently migrated on SDS-PAGE as a single band of approximately 80 kDa (Fig 2A). Rabbit polyclonal antibodies raised against rSmPOP reacted with the original rSmPOP antigen by immunoblotting and recognized a single band in the homogenates from schistosome adults (Fig 2A). The molecular mass of both the native SmPOP and rSmPOP is in good agreement with the theoretical mass of SmPOP predicted from the amino-acid sequence (82 kDa).

The pH activity profile of rSmPOP was determined using the fluorogenic substrate Z-Gly-Pro-AMC and compared with that of the native SmPOP in schistosome adult homogenates (Fig 2B). For both protein sources, the substrate was cleaved between pH 6.0 and 10.0 with optimal activity around pH 8.0. No POP activity was detected below pH 5.0.

Prolyl oligopeptidases perform specific post-proline cleavages of various peptides [39,41]. Accordingly, using a broad panel of proline-containing bioactive peptides, we asked whether SmPOP cleaves human peptide hormones and neuropeptides (Fig 3). After incubation of the

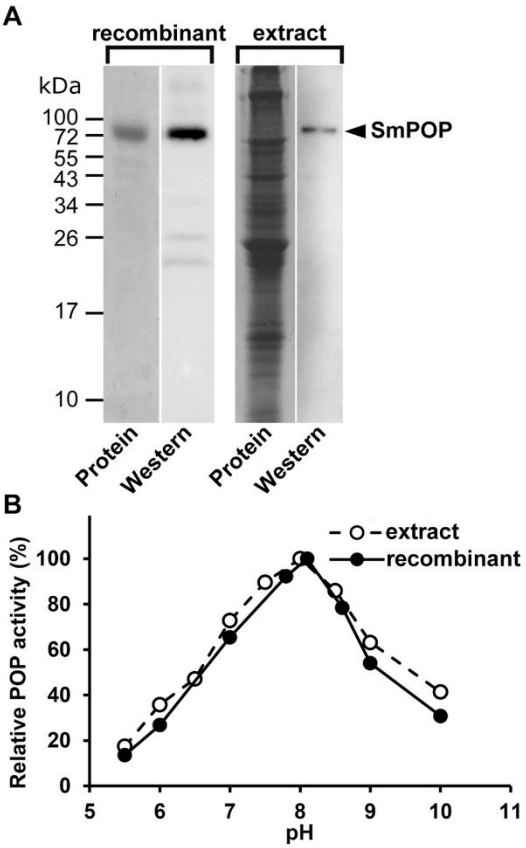

Fig 2. A comparison of recombinant SmPOP and native SmPOP. (A) Recombinant SmPOP expressed in *E. coli* (two left lanes) and *S. mansoni* protein extract (two right lanes) were resolved by SDS-PAGE, blotted onto a membrane, and visualized by protein staining or by anti-SmPOP IgG (polyclonal antibodies raised against recombinant SmPOP). (B) The pH profile of recombinant SmPOP and native SmPOP (in *S. mansoni* extracts). Activity was measured in a kinetic assay with the fluorogenic substrate Z-Gly-Pro-AMC. Mean values, expressed as a percentage are shown (the S.D. values of three replicates are within 10% of the mean).

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tested peptides with SmPOP, the resulting fragments were separated by HPLC and the cleavage positions identified by mass spectrometry. All substrates were cleaved specifically at the carboxyl terminus of proline residues with the exception of the Pro-Lys bond in Substance P and the Pro-Pro bond in bradykinin (Fig 3). The substrate specificity resembles that of mammalian prolyl oligopeptidases, which cleave a Pro-Xaa bond in peptides, where Xaa is not a Pro residue. Also, like mammalian prolyl oligopeptidases, SmPOP does not cleave after a penultimate N-terminal Pro residue [42].

The activity of rSmPOP towards host-derived macromolecular substrates was tested with several human proteins, including hemoglobin, serum albumin and collagens I and IV. No hydrolysis was observed even after prolonged incubation (S4 Fig), indicating that SmPOP is a true oligopeptidase with an action restricted to oligopeptide substrates.

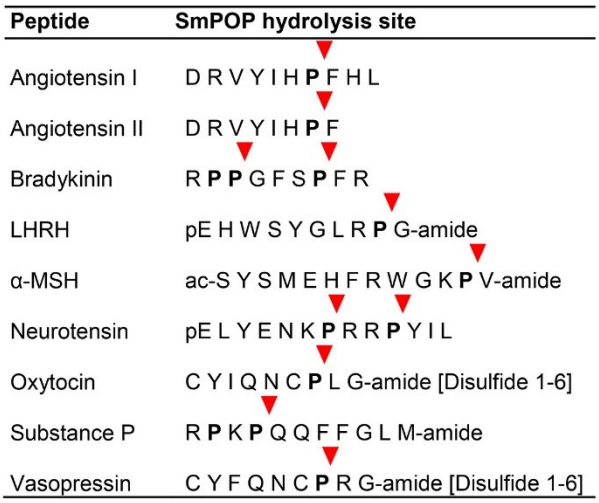

Fig 3. SmPOP cleaves human, proline–containing peptide hormones and neuropeptides. The peptides were incubated with recombinant SmPOP at pH 8.0 and the cleavage positions (the red triangles) identified by mass spectrometry. Proline residues are indicated in bold; the disulfide connectivity is indicated in parentheses.

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A fluorogenic substrate library was used to determine the SmPOP cleavage specificity at the substrate P2 position (Fig 4A). The greatest preference was recorded for basic residues (Arg and Lys), but a variety of other amino acid residues was also acceptable at this position, including hydrophobic, aliphatic and polar residues. Substrates with acidic residues and Pro at P2 were least preferred.

The substrate specificity of rSmPOP was further investigated using FRET synthetic substrates which had been designed based on the aminobenzoyl (Abz)-nitrophenylalanine (NPh) donor–acceptor pair and contained a Pro residue at P1 (Fig 4B). We prepared a set of substrates with variations in the P2 position (Abz-Ala-Pro-NPh, Abz-Gly-Pro-NPh, Abz-Lys-Pro-NPh, and Abz-Pro-Pro-NPh) and which were lengthened to include the P3 (Abz-Ala-Ala-Pro-NPh and Abz-Gly-Gly-Pro-NPh) or P1' positions (Abz-Ala-Pro-Ala-NPh and Abz-Ala-Pro-Gly-NPh). The greatest rSmPOP activity was measured with the substrates Abz-Ala-Pro-NPh and Abz-Lys-Pro-NPh, whereas the substrate Abz-Pro-Pro-NPh was not digested; increasing the substrate length to P3 and P1' positions did not increase its affinity (Fig 4B).

Finally, rSmPOP was tested for its ability to hydrolyze substrates with Pro in the P1 position that allows for cleavage by other post-proline cleaving enzymes, including collagenase-like peptidases (Suc-Gly-Pro-Leu-Gly-Pro-AMC), dipeptidyl aminopeptidase II (Lys-Pro-AMC), dipeptidyl aminopeptidase IV (Gly-Pro-AMC) and prolyl aminopeptidase (Pro-AMC; Fig 4C). Only Suc-Gly-Pro-Leu-Gly-Pro-AMC, suitable for the endopeptidase mode of cleavage, was digested by rSmPOP with the same efficiency as found for the classical and minimal POP substrate, Z-Gly-Pro-AMC. The cleavage of exopeptidase substrates with free N-termini occurs only very slowly (Lys-Pro-AMC) or not at all (Gly-Pro-AMC and Pro-AMC).

To summarize, SmPOP is a true oligopeptidase that hydrolyzes peptide but not protein substrates in the endopeptidase mode with a strict specificity for Pro at P1.

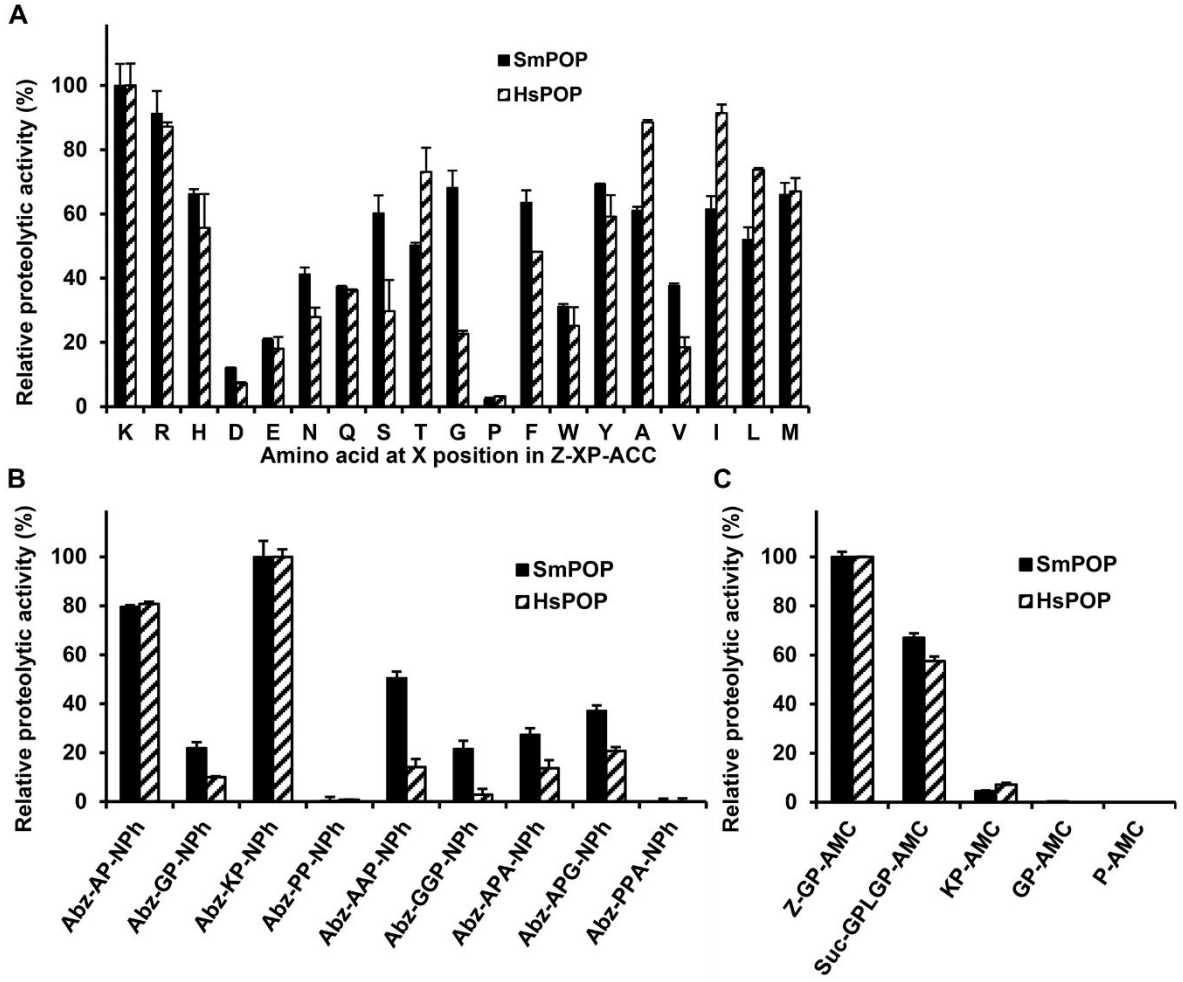

Fig 4. Substrate specificity of recombinant SmPOP. The peptidolytic activity of SmPOP was probed using a series of synthetic substrate libraries: (A) fluorogenic substrates Z-XP-ACC with proline in the P1 position and the indicated amino acids in the P2 position; (B) FRET-based peptide substrates with-XP- (in the P2 and P1 positions) extended to occupy the P3 and P1' positions; (C) fluorogenic substrates with proline in the P1 position, which w used to assay the following peptidases: collagenase-like peptidases (Suc-GPLGP-AMC), dipeptidyl aminopeptidase II (KP-AMC), dipeptidyl aminopeptidase IV (GP-AMC) and prolyl aminopeptidase (P-AMC). The substrate hydrolysis was measured in a kinetic assay at pH 8.0 using recombinant SmPOP or human POP (HsPOP). The mean values ± S.D. of three replicates are normalized to the maximum value in each series.

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Specificity of SmPOP inhibition and the design of specific inhibitors

The general inhibition specificity of rSmPOP was analyzed using a panel of peptidase class/type-selective small-molecule inhibitors as listed in Table 1. rSmPOP activity was completely inhibited by selective prolyl-oligopeptidase inhibitors with chloromethyl (CMK) and aldehyde (CHO) warheads (Z-Ala-Pro-CMK and Z-Pro-CHO), and by the general serine peptidase inhibitor, diisopropyl fluorophosphate. Partial inhibition was observed with Pefabloc SC, PMSF

Table 1. Inhibition of recombinant SmPOP by protease inhibitors.

Inhibitor[a]	Target protease[b]	Concentration (μM)	Inhibition (%)[c]
Pefabloc SC	SP	1000	12.0±3.1
PMSF	SP	1000	47.6±1.6
Benzamidine	SP (trypsin type)	10	3.7±1.1
TLCK	SP (trypsin type)	1	38.3±1.2
TPCK	SP (chymotrypsin type)	1	67.2±6.2
3,4-dichloroisocoumarin	SP	100	77.3±0.6
BPTI (Aprotinin)	SP	50	1.4±1.1
STI	SP	10	12.3±3.2
Diisopropyl fluorophosphate	SP	100	100±1
Leupeptin	SP, CP	20	2.3±1.2
Antipain	SP, CP	20	32.4±1.4
E64	CP	10	6.5±6.1
Pepstatin A	AP	1	7.3±3.5
EDTA	MP	1000	3.8±2.3
Bestatin	MP (leucin aminopeptidase)	1	2.3±2.1
Z-Ala-Pro-CMK[d]	SP (prolyl oligopeptidase)	1	100±3
Z-Pro-Pro-CHO[d]	SP (prolyl oligopeptidase)	1	100±1
Z-Pro-Pro-OH[d]	SP (prolyl oligopeptidase)	100	37.6±2.1
Z-Pro-OH[d]	SP (prolidase)	100	41.1±1.8

[a] Abbreviations: PMSF (phenylmethylsulfonyl fluoride), TLCK (Nα-Tosyl-L-lysine chloromethyl ketone), TPCK (N-p-Tosyl-L-phenylalanine chloromethyl ketone), BPTI (bovine pancreatic trypsin inhibitor), STI (soybean trypsin inhibitor), E64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane)

[b] The target proteases are classified based on catalytic type into aspartic (AP), cysteine (CP) and serine (SP) proteases, and metalloproteases (MP).

[c] The recombinant SmPOP was pre-incubated with the given inhibitor and remaining activity was measured in a kinetic assay with the fluorogenic substrate Z-Gly-Pro-AMC. The mean values ± S.D. of three replicates are expressed as percentage inhibition relative to the uninhibited control.

[d] CMK: chloromethyl ketone; CHO: aldehyde; OH: free carboxyl.

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(phenylmethylsulfonyl fluoride), TLCK (Nα-tosyl-L-lysine chloromethyl ketone), TPCK (N-p-tosyl-L-phenylalanine chloromethyl ketone) and 3,4-dichloroisocoumarin, all of which target the serine peptidases of the chymotrypsin S1 family. SmPOP activity was neither affected by protein inhibitors of serine peptidases (soybean trypsin inhibitor (STI) and bovine pancreatic trypsin inhibitor (BPTI)) nor by the inhibitors of cysteine, aspartic and metallo-peptidases. This overall inhibition profile shows that SmPOP has the ligand-binding characteristics analogous to those of mammalian POPs [41,42].

A more detailed inhibitor specificity profile for rSmPOP was investigated using a panel of synthetic peptidic inhibitors with the structure Z-Xaa-Pro-CHO/CMK, which included aldehyde (CHO) or chloromethylketone (CMK) reactive warheads (Table 2). The amino-acid residues for the Xaa position were selected based on the S2 substrate specificity of rSmPOP (Fig 4A). Table 2 shows that the synthesized aldehyde derivatives inhibit SmPOP with IC_{50} values in the low micromolar concentration range (1.3 to 6.1 μM); the inhibitory specificity at the binding subsite S2 corresponds to the substrate specificity profile (Fig 4A) and shows that inhibitors with the basic amino acids in the P2 have position have the lowest IC_{50} values.

The introduction of an irreversible covalent CMK warhead to the inhibitor scaffold improved the IC_{50} value by three orders of magnitude (IC_{50} from 2.9 to 3.2 nM) in comparison with inhibitors containing reversible covalent CHO warhead (Table 2). Furthermore, we tested the sensitivity of rSmPOP to three commercially available inhibitors developed for human POP, namely Y-29794 oxalate [43], SUAM 14746 [44], and Z-Pro-Pro-CHO [40]. Whereas the

Table 2. Inhibition of SmPOP activity and anti-schistosomal effect of synthetic SmPOP inhibitors.

Inhibitor	IC_{50} (μM)[a]		Severity of phenotype against parasite[b]
	SmPOP	HsPOP	Grade
Y-29794 oxalate[c]	8.6±0.4	0.49±0.03	II
SUAM 14746[d]	0.092±0.005	0.083±0.007	no effect
Z-Pro-Pro-CHO[e]	0.16±0.03	0.012±0.005	no effect
Z-Ala-Pro-CHO[e]	3.1±0.2	6.1±0.3	III
Z-Gly-Pro-CHO[e]	6.1±0.4	7.6±0.9	II
Z-Tyr-Pro-CHO[e]	4.4±0.4	11.4±0.7	II
Z-Arg-Pro-CHO[e]	1.3±0.3	2.4±0.2	II
Z-Lys-Pro-CHO[e]	3.0±0.6	7.2±0.6	I
Z-Ala-Pro-CMK[e]	0.0032±0.0004	0.0168±0.0046	II
Z-Arg-Pro-CMK[e]	0.0029±0.0001	0.0048±0.0006	III

[a] The IC_{50} values were determined in a kinetic activity assay with recombinant SmPOP or HsPOP and the fluorogenic substrate Z-Gly-Pro-AMC at pH 8.0. The mean values ± S.D. of three replicates are given.

[b] Induction of phenotype alterations by the inhibitors was determined with NTS in culture. The inhibitors were tested at 10 μM and 1 μM concentrations, and the resulting phenotypes, arising as a function of time and concentration, were graded I to III, with grade I being the most severe (see Materials and Methods).

[c] Y-29794 oxalate: 2-[[8-(Dimethylamino)octyl]thio]-6-(1-methylethyl)-3-pyridinyl-2-thienylmethanone oxalate

[d] SUAM 14746: 3-([4-[2-(E)-styrylphenoxy]butanoyl]-L-4-hydroxyprolyl)thiazolidine).

[e] Peptidic inhibitors with reactive aldehyde (CHO) or chloromethyl ketone (CMK) warheads (see Materials and Methods).

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inhibition by SUAM 14746 was similar for both the human and schistosomal enzymes (IC_{50} values of 83 nM and 92 nM, respectively), Y-29794 oxalate and Z-Pro-Pro-CHO inhibited SmPOP with IC_{50} values that were about one order of magnitude greater than those for human POP (IC_{50} values of 8.6 μM and 0.49 μM, respectively, for Y-29794 oxalate, and 0.16 μM and 0.01 μM, respectively, for Z-Pro-Pro-CHO).

A spatial model of SmPOP was constructed by homology modeling to study the structure-activity/inhibition relationship. The X-ray structure of porcine POP (PDB code 1QFS) was used as a template. Fig 5 shows that SmPOP has the conserved architecture of the mammalian POP comprising both the β-propeller and peptidase domains [45]. The peptidase domain (residues 430–712) has a characteristic α/β-hydrolase fold [46,47] which consists of a central eight-stranded β-sheet flanked on both sides by eight α helices. The catalytic amino-acid residues Ser556, Asp643 and His682 are located in a large cavity at the interface between the domains. The disk-shaped β-propeller domain (residues 76–429) is composed of seven repeats of four-stranded antiparallel β-sheets which are arranged around a central tunnel.

The binding mode of SmPOP was analyzed using the transition-state analog POP inhibitor Z-Pro-Pro-CHO (benzyloxycarbonyl-L-prolyl-L-prolinal) which was docked into the SmPOP active site based on the crystallographic complex of this inhibitor with porcine POP (PDB code 1QFS). The docking model (Fig 5) shows that the prolinal residue of the inhibitor forms a covalent hemi-acetal linkage with the catalytic Ser556. The P1 Pro ring binds to the hydrophobic S1 binding pocket (defined by Phe478, Trp597, Tyr601 and Val646 residues) and is stacked against a Trp597 side chain. The backbone of both the P1 and P2 proline residues forms three

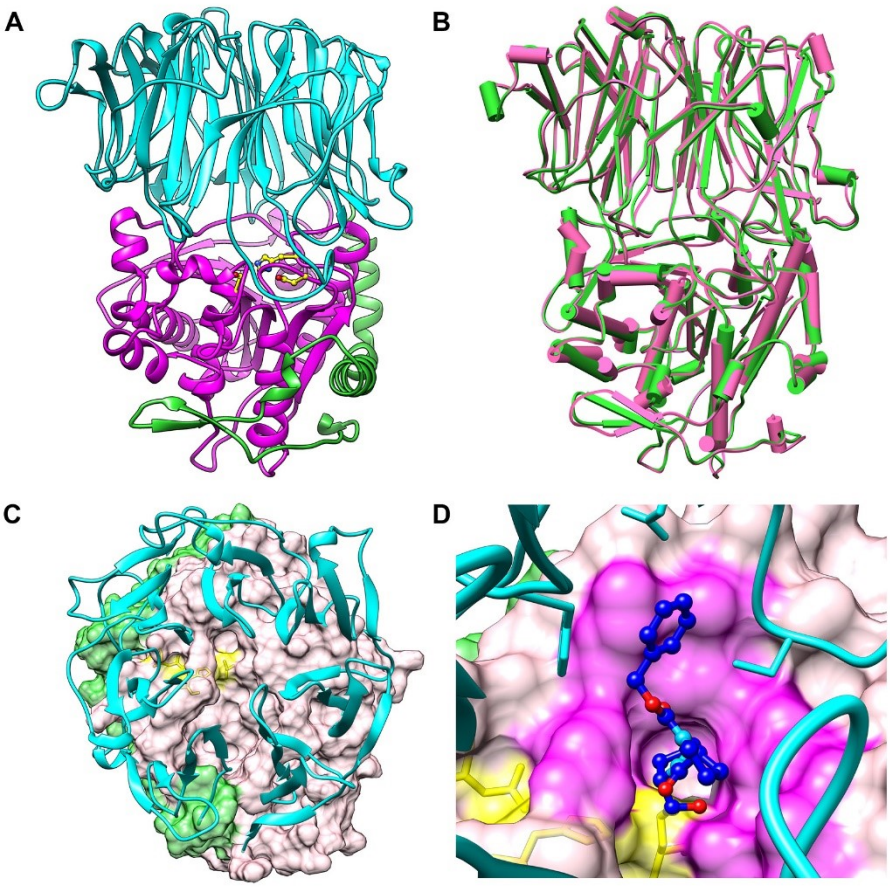

Fig 5. A three-dimensional homology model of SmPOP. (A) A ribbon representation of the overall SmPOP structure showing the β-propeller domain (cyan) and the catalytic domain (pink); the N-terminal segment is colored green. The active site containing the catalytic triad Ser556, Asp643 and His682 (yellow) is located at the interface of the two domains. (B) A superposition of the SmPOP model (green) and the porcine POP crystal structure (pink with the PDB code 1QFS) in a cylinder representation. (C) A view from the top of the SmPOP model (the β-propeller domain (cyan, ribbon representation) controls access to the active site of the catalytic domain (the pink surface) indicated by the catalytic triad residues (the yellow surface and sticks); the N-terminal segment is shown as the green surface. (D) A surface representation of the SmPOP active site located in the catalytic domain (the pink surface). The covalently-bound inhibitor Z-Pro-Pro-CHO is depicted in the ball-and-stick representation (carbon atoms in blue, oxygen in red and nitrogen in light blue). The catalytic-domain residues forming contacts with the inhibitor are highlighted as the magenta surface; the catalytic triad residues are represented by the yellow surface/sticks. The β-propeller domain is shown as a cyan ribbon, the residues interacting with the inhibitor as cyan sticks.

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hydrogen bonds to the SmPOP active site. Additionally, the P3 benzyloxycarbonyl group binds to the hydrophobic S3 binding site (residues Phe175, Cys257, Asn273, Ile593 and Ala596).

SmPOP is localized in the tegument and parenchyma of adult schistosomes

Indirect immunofluorescence microscopy on semi-thin sections using affinity-purified antibodies against rSmPOP demonstrate that SmPOP is expressed in the parenchyma and tegument of adult schistosomes (Fig 6; for a high-resolution micrograph, see Fig 7). The intensity

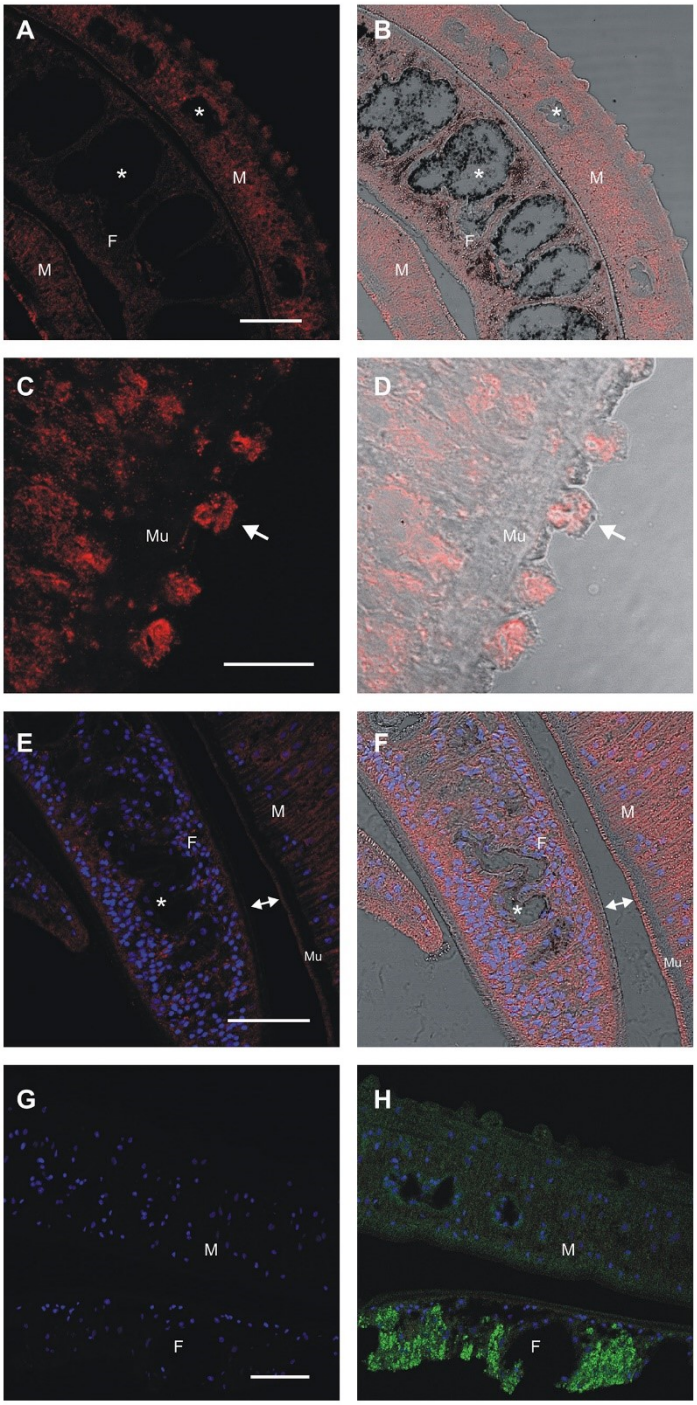
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Fig 6. SmPOP is localized to the tegument and parenchyma of adult *S. mansoni*. Semi-thin sections of adult male and female *S. mansoni* were probed with an anti-SmPOP IgG (A–F) or a pre-immune IgG (G, H) followed by reaction with an anti-rabbit IgG Alexa 594-labeled secondary antibody (red). DAPI was used to label the nuclear DNA (blue); female vitellaria are characterized by strong autofluorescence in the green spectrum (H). The left column shows merged fluorescent channels; in the right column, the signal is merged with differential interference contrast (except in H). Male worms (M) incubated with anti-SmPOP show a stronger immune-reactivity than female worms (F) (micrographs A and B). A red fluorescent signal is found in the parenchyma and tegument, but it is absent from the gut (the asterisks in A, B, E and F) and muscular tissue (Mu, micrographs C-F). In male worms the signal is found accumulated in the tubercles of the dorsal tegument (the arrows in C and D) and also outlines the gynaecophoral canal. Note the difference in signal intensity between the male and female tegument (the connected arrowheads in E and F). Only faint background fluorescence could be detected in the red spectrum in the negative control probed with pre-immune IgG (the micrographs G and H). The scale bar in C and D represents 20 μm; in A, B, E–H, 50 μm.

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of the signal was greater in the tegument of the male compared to the female (Fig 6C and 6D). Labeling was not observed in the gastrodermis, gut lumen and muscular tissues (Fig 7). Intense staining was seen in the male tegumental tubercles (Fig 6C and 6D). Pre-immune serum was applied as a negative control and only faint background fluorescence was detected (Fig 6G and 6H). Similar results were obtained in immuno-histochemical studies with NTS (S5 Fig). With this developmental stage, SmPOP was localized at or close to the surface; a low diffuse signal was also seen in the parenchyma whereas the gut exhibited no specific fluorescence. No reaction was observed with pre-immune serum (S5 Fig).

SmPOP on living parasites cleave host peptides containing proline

We investigated whether SmPOP can interact with peptidic substrates in the environment surrounding the schistosome. NTS or adult schistosomes were incubated in the presence of the

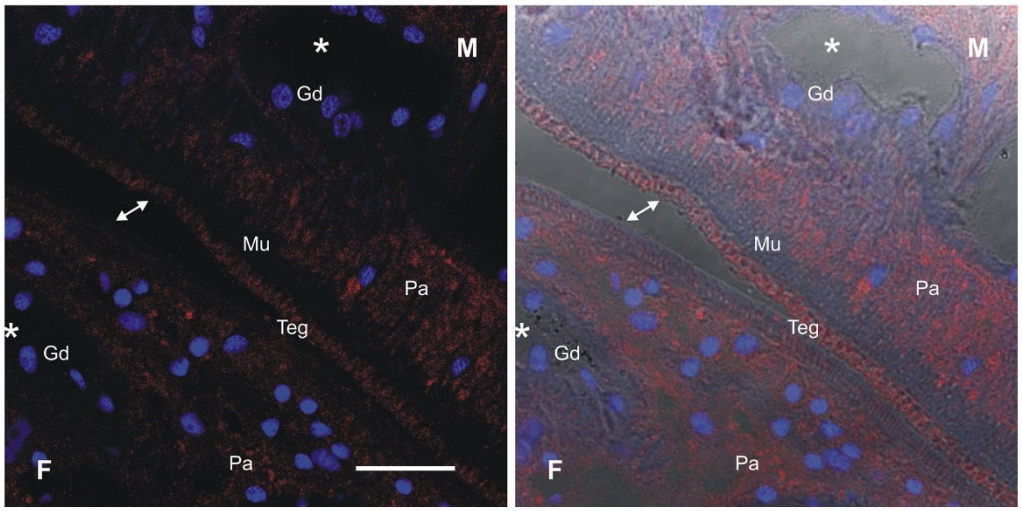

Fig 7. Detailed micrograph of SmPOP localization in the tegument of adult *S. mansoni*. The tissue section was probed with anti-SmPOP IgG followed by an anti-rabbit IgG Alexa 594-labeled secondary antibody (red). DAPI was used to label the nuclear DNA (blue). The left image shows merged fluorescent channels; on the right, the fluorescent signal is merged with differential interference contrast imaging. A red fluorescent signal is found in the parenchyma (Pa) and tegument (Teg), but is absent from the gastrodermis (Gd), gut lumen (the asterisks) and muscular tissue (Mu). Male worms (M) show a stronger immuno-reactivity than female worms (F). Note the difference in the signal intensity on the tegument of the male compared to the female (the connected arrowheads). Scale bar = 20 μm.

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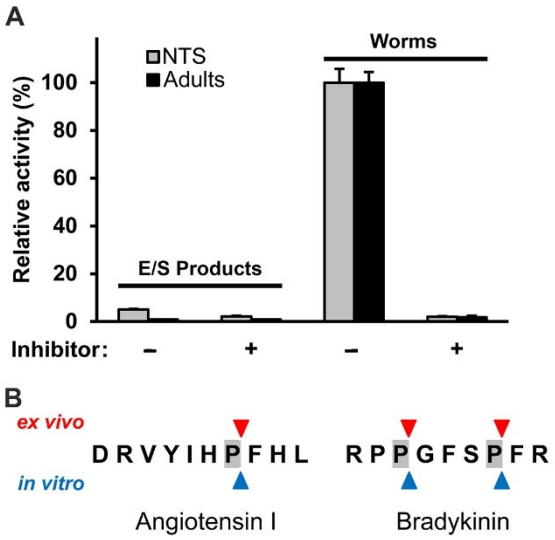

Fig 8. SmPOP in live *S. mansoni* cleaves vasoregulatory hormones. (A) SmPOP activity detected in the excretory-secretory products of or associated with live NTS and adults (worms) maintained in culture was determined using the fluorogenic substrate Z-GP-AMC. The inhibitor, Z-Ala-Pro-CMK, was added in the control experiments to specifically block SmPOP activity. The mean values ± S`.D. of three replicates are normalized to the maximum value in each series. (B) The peptide hormones angiotensin I and bradykinin were incubated with recombinant SmPOP (*in vitro*) or with live adults maintained in culture (*ex vivo*). The reaction mixture and cultivation medium, respectively, were analyzed by mass spectrometry and cleavage positions (triangles) in the hormones were identified (see also S3 Table).

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fluorogenic peptide substrate Z-Gly-Pro-AMC. Cleavage of the substrate was measured in a microplate reader and was abolished in the presence of the specific POP inhibitor Z-Ala-Pro-CMK (Fig 8A). We also tested whether SmPOP activity is measurable in the excretory/secretory (E/S) products of NTS and adults. For this purpose, E/S products were collected after a two-day cultivation of parasites and SmPOP activity was measured using the same fluorogenic substrate. No significant POP activity was detected in E/S products, demonstrating that SmPOP is not secreted into the cultivation media.

In the next step, we used the above culture assay to measure cleavage by adult parasites of two vasoregulatory proline-containing hormones from the human host, namely angiotensin I and bradykinin. Both hormones were cleaved when added to the cultivation medium and the cleavage occurred specifically after Pro residues as demonstrated by mass spectrometry (Fig 8B). Again, the fragmentation was abolished in the presence of a POP-specific inhibitor Z-Ala-Pro-CMK (but not in the presence of the cysteine peptidase inhibitor E-64; S3 Table). The identified cleavage positions in the hormone sequences were identical with those obtained by *in vitro* fragmentation using rSmPOP.

To conclude, SmPOP, although not secreted from the parasite, can nonetheless interact with physiologically relevant host peptides in the environment.

SmPOP inhibitors induce deleterious phenotypes in cultured schistosomula

A panel of SmPOP inhibitors was tested at 1 and 10 μM against NTS and the phenotypic responses graded I through III from the most to the least severe (Table 2). The CHO inhibitor, Z-Lys-Pro-CHO, induced a grade I response. Grade II phenotypes were induced by Z-Gly-Pro-CHO, Z-Tyr-Pro-CHO, Z-Arg-Pro-CHO and the CMK inhibitor, Z-Arg-Pro-CMK. The inhibitors Z-Ala-Pro-CHO and Z-Ala-Pro-CMK induced the least severe grade III phenotype. The commercial inhibitors of human POP, Y-29794 and SUAM 14746, induced a grade II response or had no effect, respectively (Table 2).

Discussion

We identified and functionally characterized a S9-family serine peptidase from the human blood fluke, *S. mansoni*. It was denoted SmPOP, *S. mansoni* prolyl oligopeptidase, based on its 51% primary sequence identity to human and porcine prolyl oligopeptidases. Also, homology modeling of SmPOP using porcine POP as a structural template revealed that both enzymes share the same spatial architecture and domain structure; specifically, a catalytic peptidase domain with an α/β hydrolase fold and a catalytic triad, and a cylindrical β-propeller domain that covers the active site and defines prolyl oligopeptidase as an oligopeptidase [48].

SmPOP was heterologously expressed in *E. coli*, purified as an active peptidase and subjected to a series of biochemical analyses to determine its substrate and inhibitory specificity. Consistent with its classification as a S9-family prolyl oligopeptidase, the enzyme cleaves various oligopeptide substrates in an endopeptidolytic mode at the carboxyl terminus of Pro residues [45]. Cleavage specificity analysis with the positional-scanning substrate library revealed a preference for basic amino acids over hydrophobic or aliphatic amino acids at P2; a Pro residue at P2 was unfavorable. A similar S2 subsite specificity profile was obtained for human POP (Fig 4).

rSmPOP was effectively inhibited by the general serine peptidase inhibitor, diisopropylfluorophosphate [49], but only weakly by inhibitors targeting the S1 family of serine peptidases such as Pefabloc, benzamidine, and BPTI. These data are consistent with the inhibitory specificities of mammalian and trypanosomal POPs [50,51]. The inhibitor specificity of rSmPOP was investigated further using a panel of synthetic prolinal inhibitors that vary at the P2 amino-acid residue (Z-Xaa-Pro-CHO, Table 2). The inhibitor specificity profile mirrored that determined with the positional- scanning substrate library, with the exception of the Pro residue in the P2 position, which generates a good inhibitor but a poor substrate (Z-Pro-Pro-CHO vs. Z-Pro-Pro-ACC, respectively, Table 2 and Fig 4A). Note that Z-Pro-Pro-ACC substrate does not bind effectively in the active site neither as the uncleaved form nor as the hypothetical cleavage product Z-Pro-Pro-OH (as they do not compete with Z-Gly-Pro- substrate). A similar discrepancy was observed for human POP (Table 2 and Fig 4A). Based on the assembled biochemical and structural data, therefore, it is clear that SmPOP and its mammalian orthologs are almost identical in their catalytic specificity profiles suggesting a strong evolutionary conservation of function and structure.

The panel of SmPOP inhibitors was further evaluated for their anti-schistosomal effects against NTS in culture. These tests demonstrated that some of the investigated inhibitors induced deleterious phenotypes or death. Although, interactions other than with the specific target protein cannot be excluded, the data encourage the search for small molecule inhibitors of SmPOP. Inhibitors of human POP are currently being examined as drug leads in several neurological disorders such as depression, Alzheimer's disease and amnesia, and a number are in preclinical and clinical trials as nootropics (for review see [44]). POP is also of interest for the treatment of celiac sprue, an inflammatory disease of the small intestine caused by ingesting proline-rich gluten [39].

The prolyl oligopeptidases Tc80 and Tb80 from the protozoan parasites *Trypanosoma cruzi* and *T. brucei*, respectively, are secreted and can degrade host extracellular-matrix (ECM) proteins such as proline-rich collagens I and IV [23,51]. Tc80's ability to ability to degrade of ECM components contributes directly to the invasion of mammalian cells by *T. cruzi* trypomastigotes [22]. In contrast, SmPOP cannot degrade protein substrates, including collagens, even though it has about 40% identity with trypanosomal POPs (S1 Table). Further, SmPOP is not found in *S. mansoni* E/S products suggesting that it is not secreted by schistosomes, a finding consistent with the absence of the signal peptide in the SmPOP sequence. The data would therefore indicate that the trypanosomal POPs possess different physiological functions from those postulated below for the schistosomal enzyme.

By RT-qPCR and substrate analysis, SmPOP is expressed in those developmental stages parasitizing the definitive mammalian host (adults, NTS and eggs). By immunolocalization with a monospecific rabbit antibody SmPOP is distributed in the tegument (males) and parenchyma of NTS and adult schistosomes. The enzyme is absent from the gastrodermis and gut lumen suggesting that the enzyme does not contribute to the digestion of ingested blood proteins. The antibody signal was significantly greater in male worms in accordance with the activity profiling of worm extracts, whereby male worm extracts displayed 5–6 times greater SmPOP specific enzymatic activity than females (S6 Fig). Intriguingly, SmPOP is found in the male tegument, not least in the tubercles, but is apparently absent from the female tegument. This suggests that SmPOP may have male-specific peptidolytic functions at the host–parasite interface and/or at the male-female interface. As noted above, the enzyme seems not to be secreted by the parasite yet, via contact with the endothelium of the host vasculature, may exert localized effects on vascular physiology, including the degradation of vasoactive peptides (see below). A similar localization in the tegument and parenchyma was previously noted for the *S. mansoni* cysteine peptidase cathepsin B2 for which physiological function(s) are not yet known [52].

We demonstrate that the schistosome parasite can cleave the vasoregulatory peptides, angiotensin I and bradykinin, when co-incubated *in vitro* and that the activity is due to SmPOP as indicated by mass spectrometry and specific inhibition by a POP inhibitor. Angiotensin I is produced by the renin-angiotensin system which is the primary physiological regulator of blood pressure, sodium balance and fluid volume [53]. SmPOP converts angiotensin I (precursor of the main vasoconstrictor angiotensin II) to the vasodilatory angiotensin-(1–7). Angiotensin-(1–7) also inhibits cell proliferation, angiogenesis, fibrosis, and inflammation [54,55]. Bradykinin is generated by the kallikrein-kinin system which also participates in the regulation of blood pressure [53]. Bradykinin is a potent vasodilator, promotes natriuresis, diuresis and inflammation. Proteolytic cleavage by SmPOP inactivates this hormone. The possible contribution, therefore, by a tegument-localized SmPOP to the modulation or dysregulation of both these, and possibly, other, homeostatic systems is conceivable whereby cleavage of the pro-inflammatory and vasoconstrictory angiotensin I and pro-inflammatory bradykinin may provide a survival benefit to the schistosome during its residence in and movement through the venous blood system. Follow-up *in vivo* studies will examine these possibilities in more detail.

Supporting Information

S1 Fig. Examples of phenotypes induced in cultured NTS by POP inhibitors listed in Table 2. NTS were incubated up to four days in Basch Medium 169 in the presence of inhibitors (for details see Methods). Images were captured using a Zeiss Axiovert 40 C inverted microscope (10x objective) and a Zeiss AxioCam MRc digital camera controlled by AxioVision 40 (version 4.8.1.0) software. Scale bar = 150 μm.
(TIF)

S2 Fig. A multiple sequence alignment of *S. mansoni* prolyl oligopeptidase (SmPOP) with selected POPs from other blood-feeding parasites and mammalian POPs. Parasite POPs: SmPOP (S. mansoni, GenBank accession number KF956809), *Pediculus humanus* (P. humanus, XP_002430998), *Aedes aegypti* (A. aegypti, Q16WP2), *Ixodes scapularis* (I. scapularis, B7PDF5), *Toxoplasma gondi* (T. gondi, XP_002369249), *Trypanosoma cruzi* (T. cruzi, AAQ04681) and *Leishmania infantum* (L. infantum, CAM72491.1). Mammalian POPs: human (H. sapiens, P48147) and porcine (S. scrofa, P23687). Catalytic-triad residues (Ser, Asp and His) are indicated in red; those residues identical with those of SmPOP are shaded in gray. The residue numbering corresponds to the SmPOP sequence and its color coding refers to the domain structure of POPs consisting of the N-terminal segment (green), the β-propeller domain (cyan) and the peptidase catalytic domain (magenta).
(TIF)

S3 Fig. The maximum-likelihood phylogenetic tree displaying the evolutionary relationship of SmPOP to selected POPs from other organisms. A multiple alignment of SmPOP with 35 other POP protein sequences was performed using Clustal X 2.0 and the default parameters. The resulting alignment was edited to exclude ambiguous regions by the BioEdit 7.0 editing software. The phylogenetic analysis of the multiple alignment was performed using the maximum likelihood method in PAUP 4.0. The tree was visualized using the Treeview 1.6.6. program. Bootstrap values with 100 repeats are shown at the nodes. GenBank or HelmDB accession numbers of the aligned sequences are indicated. SmPOP is underlined in red and bold type faces. **GenBank accession numbers: Kinetoplastida—**CAM72491 *Leishmania infantum*, *AAQ04681 Trypanosoma cruzi*, CAD42967 *Trypanosoma brucei; Apicomplexa—*XP_002369249 *Toxoplasma gondii*; **Bacteria—**WP_007903716 *Ktedonobacter racemifer*, WP_008080757 *Vibrio sinaloensis*, WP_012088900 *Shewanella baltica*, WP_013626821 *Planctomyces brasiliensis*, WP_011140683 *Gloeobacter violaceus*, WP_011612632 *Trichodesmium erythraeum*, EHJ13806 *Crocosphaera watsonii*, WP_012411436 *Nostoc punctiforme*, WP_006634458 *Microcoleus vaginatus*; **Arthropoda—**XP_002430998 *Pediculus humanus*, B7PDF5 *Ixodes scapularis*, Q16WP2 *Aedes aegypti*, B0W4N7 *Culex quinquefasciatus*, EFN76622 *Harpegnathos saltator*, EFN66352 *Camponotus floridanus;* **Plantae—**A9SA32 *Physcomitrella patens*, XP_002285910 *Vitis vinifera*, ACG43067 *Zea mays*; **Vertebrata—**C0HBI8 *Salmo salar*, Q503E2 *Danio rerio*, Q6P4W3 *Xenopus tropicalis*, F1NUS2 *Gallus gallus*, O70196 *Rattus norvegicus*, Q9QUR6 *Mus musculus*, F1PHX2 *Canis familiaris*, P48147 *Homo sapiens*, Q9XTA2 *Bos taurus*, P23687 *Sus scrofa;* **Trematoda—**KF956809 Schistosoma mansoni. **HelmDB accession numbers: Nematoda—**Asuu161668 *Ascaris suum;* **Trematoda**—Fhep110926 *Fasciola hepatica*, Shae172866 *Schistosoma haematobium*.
(TIF)

S4 Fig. Recombinant SmPOP does not digest host-derived macromolecular protein substrates. Human serum albumin (HSA), human collagens type I and IV (Col I and Col IV) and human hemoglobin (Hb) were incubated for 12 h in the presence or absence of rSmPOP. The reaction mixtures were subjected to SDS-PAGE (HSA, Col I and Col IV) or Tricine-SDS-PAGE (Hb) and protein stained. For details, see Methods.
(TIF)

S5 Fig. The localization of SmPOP in *S. mansoni* newly transformed schistosomula (NTS). Parasites were fixed and probed with anti-SmPOP IgG (A) or a pre-immune IgG from the same rabbit (B). Anti-rabbit IgG Alexa 594-was used as the secondary antibody (red). DAPI was used to label the nuclear DNA (blue) and the fluorescent signals were merged with differential interference contrast (DIC). The greatest red fluorescence is localized to the surface (tegument) with a low diffuse signal in the parenchyma (SmPOP in A). The gut is negative for the

SmPOP signal (the asterisk). NTS probed with pre-immune IgG lack any visible fluorescence in the red channel (SmPOP in B). Scale bar = 50 μm.
(TIF)

S6 Fig. The SmPOP activity in *S. mansoni* adult males and females. SmPOP activities were measured in protein extracts of adult males and females (green bars) or in cultivation medium post incubation with live parasites (red bars). Z-Gly-Pro-AMC was used as the fluorogenic substrate. SmPOP activity (which was sensitive to inhibition by the specific POP inhibitor, Z-Pro-Pro-CHO) was normalized to protein content of extracts or number of worms used.
(TIF)

S1 Table. Identity matrix of POP amino acid sequences aligned in S1 Fig.
(PDF)

S2 Table. Identification of native SmPOP by mass spectrometry.
(PDF)

S3 Table. The fragmentation of peptide hormones by live *S. mansoni* adults.
(PDF)

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Author Contributions

Conceived and designed the experiments: SS JD JV CRC MM MHo. Performed the experiments: PF SS MHr JD JV AJ LU CRC MHo. Analyzed the data: PF SS MHr JD JV CRC MM MHo. Contributed reagents/materials/analysis tools: SS MHr JD JV JHM CRC MM MHo. Wrote the paper: PF SS JD JHM CRC MM MHo.

References

1. Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J (2006) Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect Dis 6: 411–425. S1473-3099(06)70521-7. PMID: 16790382
2. Lockyer AE, Olson PD, Ostergaard P, Rollinson D, Johnston DA, et al. (2003) The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of Schistosoma Weinland, 1858. Parasitology 126: 203–224. PMID: 12666879
3. Gryseels B, Polman K, Clerinx J, Kestens L (2006) Human schistosomiasis. Lancet 368: 1106–1118. PMID: 16997665
4. Burke ML, Jones MK, Gobert GN, Li YS, Ellis MK, et al. (2009) Immunopathogenesis of human schistosomiasis. Parasite Immunol 31: 163–176. doi: 10.1111/j.1365-3024.2009.01098.x PMID: 19292768
5. Crum NF, Chun HM, Favata MA, Hale BR (2003) Gastrointestinal *Schistosomiasis japonicum* infections in immigrants from the Island of Leyte, Philippines. J Travel Med 10: 131–132. PMID: 12650659
6. Caffrey CR (2007) Chemotherapy of schistosomiasis: present and future. Curr Opin Chem Biol 11: 433–439. PMID: 17652008
7. Melman SD, Steinauer ML, Cunningham C, Kubatko LS, Mwangi IN, et al. (2009) Reduced susceptibility to praziquantel among naturally occurring Kenyan isolates of *Schistosoma mansoni*. PLoS Negl Trop Dis 3: e504. doi: 10.1371/journal.pntd.0000504 PMID: 19688043
8. Abdulla MH, Ruelas DS, Wolff B, Snedecor J, Lim KC, et al. (2009) Drug discovery for schistosomiasis: hit and lead compounds identified in a library of known drugs by medium-throughput phenotypic screening. PLoS Negl Trop Dis 3: e478. doi: 10.1371/journal.pntd.0000478 PMID: 19597541

9. Abdulla MH, Lim KC, Sajid M, McKerrow JH, Caffrey CR (2007) Schistosomiasis mansoni: novel chemotherapy using a cysteine protease inhibitor. PLoS Med 4: e14. PMID: 17214506

10. Sajid M, McKerrow JH (2002) Cysteine proteases of parasitic organisms. Mol Biochem Parasitol 120: 1–21. PMID: 11849701

11. McKerrow JH, Caffrey C, Kelly B, Loke P, Sajid M (2006) Proteases in parasitic diseases. Annu Rev Pathol 1: 497–536. PMID: 18039124

12. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, et al. (2009) The genome of the blood fluke *Schistosoma mansoni*. Nature 460: 352–358. doi: 10.1038/nature08160 PMID: 19606141

13. Ingram JR, Rafi SB, Eroy-Reveles AA, Ray M, Lambeth L, et al. (2012) Investigation of the proteolytic functions of an expanded cercarial elastase gene family in *Schistosoma mansoni*. PLoS Negl Trop Dis 6: e1589. doi: 10.1371/journal.pntd.0001589 PMID: 22509414

14. Delcroix M, Sajid M, Caffrey CR, Lim KC, Dvorak J, et al. (2006) A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. J Biol Chem 281: 39316–39329. PMID: 17028179

15. Caffrey CR, McKerrow JH, Salter JP, Sajid M (2004) Blood 'n' guts: an update on schistosome digestive peptidases. Trends Parasitol 20: 241–248. PMID: 15105025

16. Jilkova A, Rezacova P, Lepsik M, Horn M, Vachova J, et al. (2011) Structural basis for inhibition of cathepsin B drug target from the human blood fluke, *Schistosoma mansoni*. J Biol Chem 286: 35770–35781. doi: 10.1074/jbc.M111.271304 PMID: 21832058

17. Horn M, Jilkova A, Vondrasek J, Maresova L, Caffrey CR, et al. (2011) Mapping the pro-peptide of the *Schistosoma mansoni* cathepsin B1 drug target: modulation of inhibition by heparin and design of mimetic inhibitors. ACS Chem Biol 6: 609–617. doi: 10.1021/cb100411v PMID: 21375333

18. Fanfrlik J, Brahmkshatriya PS, Rezac J, Jilkova A, Horn M, et al. (2013) Quantum mechanics-based scoring rationalizes the irreversible inactivation of parasitic *Schistosoma mansoni* cysteine peptidase by vinyl sulfone inhibitors. J Phys Chem B 117: 14973–14982. doi: 10.1021/jp409604n PMID: 24195769

19. Jilkova A, Horn M, Rezacova P, Maresova L, Fajtova P, et al. (2014) Activation route of the *Schistosoma mansoni* cathepsin B1 drug target: structural map with a glycosaminoglycan switch. Structure 22: 1786–1798. doi: 10.1016/j.str.2014.09.015 PMID: 25456815

20. Rawlings ND, Waller M, Barrett AJ, Bateman A (2014) MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 42: D503–D509. doi: 10.1093/nar/gkt953 PMID: 24157837

21. Szeltner Z, Polgar L (2008) Structure, function and biological relevance of prolyl oligopeptidase. Curr Protein Pept Sci 9: 96–107. PMID: 18336325

22. Grellier P, Vendeville S, Joyeau R, Bastos IM, Drobecq H, et al. (2001) *Trypanosoma cruzi* prolyl oligopeptidase Tc80 is involved in nonphagocytic mammalian cell invasion by trypomastigotes. J Biol Chem 276: 47078–47086. PMID: 11598112

23. Bastos IM, Grellier P, Martins NF, Cadavid-Restrepo G, de Souza-Ault MR, et al. (2005) Molecular, functional and structural properties of the prolyl oligopeptidase of *Trypanosoma cruzi* (POP Tc80), which is required for parasite entry into mammalian cells. Biochem J 388: 29–38. PMID: 15581422

24. Bastos IM, Motta FN, Grellier P, Santana JM (2013) Parasite prolyl oligopeptidases and the challenge of designing chemotherapeuticals for Chagas disease, leishmaniasis and African trypanosomiasis. Curr Med Chem 20: 3103–3115. PMID: 23514419

25. Horn M, Fajtova P, Rojo AL, Ulrychova L, Bartosova-Sojkova P, et al. (2014) Trypsin- and Chymotrypsin-Like Serine Proteases in *Schistosoma mansoni*—'The Undiscovered Country'. PLoS Negl Trop Dis 8: e2766. doi: 10.1371/journal.pntd.0002766 PMID: 24676141

26. Stefanic S, Dvorak J, Horn M, Braschi S, Sojka D, et al. (2010) RNA interference in Schistosoma mansoni schistosomula: selectivity, sensitivity and operation for larger-scale screening. PLoS Negl Trop Dis 4: e850. doi: 10.1371/journal.pntd.0000850 PMID: 20976050

27. Colley DG, Wikel SK (1974) *Schistosoma mansoni*: simplified method for the production of schistosomules. Exp Parasitol 35: 44–51. PMID: 4815018

28. Basch PF (1981) Cultivation of Schistosoma mansoni in vitro. I. Establishment of cultures from cercariae and development until pairing. J Parasitol 67: 179–185. PMID: 7241277

29. Le TH, Blair D, Agatsuma T, Humair PF, Campbell NJ, et al. (2000) Phylogenies inferred from mitochondrial gene orders-a cautionary tale from the parasitic flatworms. Mol Biol Evol 17: 1123–1125. PMID: 10889225

30. Consortium SjGSaFA (2009) The Schistosoma japonicum genome reveals features of host-parasite interplay. Nature 460: 345–351. doi: 10.1038/nature08140 PMID: 19606140

31. Young ND, Jex AR, Li B, Liu S, Yang L, et al. (2012) Whole-genome sequence of Schistosoma haematobium. Nat Genet 44: 221–225. doi: 10.1038/ng.1065 PMID: 22246508

32. Doleckova-Maresova L, Pavlik M, Horn M, Mares M (2005) De novo design of alpha-amylase inhibitor: a small linear mimetic of macromolecular proteinaceous ligands. Chem Biol 12: 1349–1357. PMID: 16356852

33. Maly DJ, Leonetti F, Backes BJ, Dauber DS, Harris JL, et al. (2002) Expedient solid-phase synthesis of fluorogenic protease substrates using the 7-amino-4-carbamoylmethylcoumarin (ACC) fluorophore. J Org Chem 67: 910–915. PMID: 11856036

34. Powers JC, Wilcox PE (1970) Design and synthesis of inhibitors for crystallographic studies on the active site of chymotrypsin. J Am Chem Soc 92: 1782–1783. PMID: 5418458

35. Ede NJ, Eagle SN, Wickham G, Bray AM, Warne B, et al. (2000) Solid phase synthesis of peptide aldehyde protease inhibitors. Probing the proteolytic sites of hepatitis C virus polyprotein. J Pept Sci 6: 11–18. PMID: 10674715

36. Caffrey CR, Ruppel A (1997) Cathepsin B-like activity predominates over cathepsin L-like activity in adult *Schistosoma mansoni* and *S. japonicum*. Parasitol Res 83: 632–635. PMID: 9211519

37. Labute P (2008) The generalized Born/volume integral implicit solvent model: estimation of the free energy of hydration using London dispersion instead of atomic surface area. J Comput Chem 29: 1693–1698. doi: 10.1002/jcc.20933 PMID: 18307169

38. Protasio AV, Tsai IJ, Babbage A, Nichol S, Hunt M, et al. (2012) A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis 6: e1455. doi: 10.1371/journal.pntd.0001455 PMID: 22253936

39. Cunningham DF, O'Connor B (1997) Proline specific peptidases. Biochim Biophys Acta 1343: 160–186. PMID: 9434107

40. Yoshimoto T, Kawahara K, Matsubara F, Kado K, Tsuru D (1985) Comparison of inhibitory effects of prolinal-containing peptide derivatives on prolyl endopeptidases from bovine brain and Flavobacterium. J Biochem 98: 975–979. PMID: 3908451

41. Garcia-Horsman JA, Mannisto PT, Venalainen JI (2007) On the role of prolyl oligopeptidase in health and disease. Neuropeptides 41: 1–24. PMID: 17196652

42. Wilk S (1983) Prolyl endopeptidase. Life Sci 33: 2149–2157. PMID: 6358755

43. Nakajima T, Ono Y, Kato A, Maeda J, Ohe T (1992) Y-29794—a non-peptide prolyl endopeptidase inhibitor that can penetrate into the brain. Neurosci Lett 141: 156–160. PMID: 1436628

44. Lawandi J, Gerber-Lemaire S, Juillerat-Jeanneret L, Moitessier N (2010) Inhibitors of prolyl oligopeptidases for the therapy of human diseases: defining diseases and inhibitors. J Med Chem 53: 3423–3438. doi: 10.1021/jm901104g PMID: 20058865

45. Polgar L (2002) The prolyl oligopeptidase family. Cell Mol Life Sci 59: 349–362. PMID: 11915948

46. Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F, et al. (1992) The alpha/beta hydrolase fold. Protein Eng 5: 197–211. PMID: 1409539

47. Nardini M, Dijkstra BW (1999) Alpha/beta hydrolase fold enzymes: the family keeps growing. Curr Opin Struct Biol 9: 732–737. PMID: 10607665

48. Fulop V, Bocskei Z, Polgar L (1998) Prolyl oligopeptidase: an unusual beta-propeller domain regulates proteolysis. Cell 94: 161–170. PMID: 9695945

49. Schonlein C, Heins J, Barth A (1990) Purification and characterization of prolyl endopeptidase from pig brain. Biol Chem Hoppe Seyler 371: 1159–1164. PMID: 2090162

50. Sharma KK, Ortwerth BJ (1994) Purification and characterization of prolyl oligopeptidase from bovine lens. Exp Eye Res 59: 107–115. PMID: 7835390

51. Bastos IM, Motta FN, Charneau S, Santana JM, Dubost L, Augustyns K, Grellier P (2010) Prolyl oligopeptidase of Trypanosoma brucei hydrolyzes native collagen, peptide hormones and is active in the plasma of infected mice. Microbes Infect 12: 457–466. doi: 10.1016/j.micinf.2010.02.007 PMID: 20188209

52. Caffrey CR, Salter JP, Lucas KD, Khiem D, Hsieh I, et al. (2002) SmCB2, a novel tegumental cathepsin B from adult Schistosoma mansoni. Mol Biochem Parasitol 121: 49–61. PMID: 11985862

53. Campbell DJ (2003) The renin-angiotensin and the kallikrein-kinin systems. Int J Biochem Cell Biol 35: 784–791. PMID: 12676165

54. Izumi Y, Iwao H (2006) Angiotensin II and Its Related Peptides. In: Katin AJ, editor. Handbook of Biologically Active Peptides, pp. 1169–1174.

55. Gallagher PE, Arter AL, Krishnan B, Garcia-Espinosa MA, Tallant A (2013) Angiotensin II/Angiotensin-(1–7). In: Katin AJ, editor. Handbook of Biologically Active Peptides. pp. 494–501.